ANALYTICAL STUDIES ON HONEY

A Thesis submitted to the University of Salford
in candidature for the Degree of

Doctor of Philosophy

by

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To UMA - for her inspiration.

DECLARATION

I hereby declare that the work contained herein, now submitted as a thesis for the degree of Doctor of Philosophy of the University of Salford is the result of my own investigations. The work was carried out in the Department of Biological Sciences, Biochemistry Section, University of Salford, under the supervision of Dr. R. J. Washington and Dr. L. R. Croft.

I certify that this work has not been submitted for any other degree and that it is not being currently presented for any other degree.

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C O N T E N T S

ABSTRACT

The floral and the geographical origins of honey can be determined by microscopic examination of the pollen present, however this technique cannot be applied to highly strained or pollen free honeys. Thus, there is a need for a chemical technique that can be applied to such samples. This present work was undertaken with the help of the British Beekeepers Association and their members, who provided honey samples of known origin within the United Kingdom for chemical evaluation and comparison.

There were 192 samples of English honey from the U.K. survey and an additional 69 samples including those of foreign origin obtained from various sources.

A selection of 11 foreign and 5 English honeys were examined for proteins present by the technique of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. This approach was abandoned since preliminary studies indicated that a correlation between the proteins and the geographical origin was not possible.

For 256 samples out of the 261, the free amino acids and the pollens present were examined.

The free ninhydrin positive substances were separated by ion-exchange chromatography and then concentrated. Forty ninhydrin positive substances were detected by a combination of paper electrophoresis and chromatography and 28 of these compounds were identified. Quantitative measurements of the concentration of 13 detectable amino acids were made on an automatic amino acid analyser. The average concentration of the 13 amino acids of the 191 samples of the U.K. survey and those of the 5 predominant pollens of the U.K. have been given in Table A.

TABLE A

Average concentration of thirteen free amino acids

Amino acids \ Sources-floral	Average concentration (nMoles per g of honey)					
	1	2	3	4	5	6
Lysine	123	170	122	78	107	133
Aspartic acid	90	167	170	124	146	136
Threonine	63	86	110	42	60	92
Serine	70	120	112	62	90	100
Glutamic acid	144	335	298	126	220	246
Proline	2,000	4,090	3,250	1,870	3,230	3,140
Glycine	44	76	100	46	66	67
Alanine	73	150	132	70	114	118
Valine	46	76	65	33	82	66
Isoleucine	43	107	62	30	70	67
Leucine	40	92	80	33	58	60
Tyrosine	55	238	100	120	75	120
Phenylalanine	170	312	180	110	218	208
Sample Number	44	29	17	8	8	191

1 = *Brassica*

2 = *Trifolium repens*

3 = *Castanea*

4 = *Calluna*

5 = *Myosotis*

6 = U.K. survey

The major floral sources of each honey were confirmed by quantitative pollen analysis of the original sample.

It was found that by performing discriminant analysis using the computer package Statistical Package for Social Sciences (SPSS) that the amino acid concentration measurements could be correlated to the floral source of the samples examined, provided the sub-group samples were sufficiently large. The overall pattern of floral sources coupled with the detection of ninhydrin positive substances can be used successfully to categorise unspecified samples. This is then followed by correlation, in some cases, with amino acid concentration measurements using the SPSS to predict a definite geographical region.

The potential role of using the SPSS predictive classifications to evaluate the following aspects of honey production were indicated to be successful. These aspects were: honeydew and nectar honeys, feeding sugar to honeybees, the effects of heating honey, adulteration especially with foreign honeys and/or commercial sugar products, commercial processing methods and floral source with reference to plant family.

I N T R O D U C T I O N

CHAPTER 1

I N T R O D U C T I O N - H O N E Y

CHAPTER 1

The definition of honey according to Pearson (1976) is: 'Honey is the saccharine product gathered by the bees from the nectar of flowers'. This definition is in agreement with those regulations issued by the United Kingdom and the European Economic Community.

Inter-relationship of species of honeybees and their products

There have been several entomological studies on the honeybee and one such investigation was by Richards and Daves (1977). Within the order of *Hymenoptera* representing those insects with membranous wings there is the *Aculeata* family. The ants, bees and wasps are members of this family and are characterised by having their ovipositor modified to form a sting. This family includes three *genii*, the *Apis*, *Bombidea* and *Melipona*. The *Apis* genus has four species of honeybees and these are the *Apis cerena*, the *Apis dorsata*, the *Apis florea* and the well-known *Apis mellifera*. The *Bombidea* genus includes the bumblebee *(Bombus)* species. The *Melipona* genus or the stingless bees as they are commonly known consist of two genera and these are the *Trigona* and the *Melipona*.

In the present text unless stated otherwise the honeybee referred to will be the *Apis mellifera*.

Analytical data on the honey obtained from the four *Apis* species and also one *Trigona* according to various literature sources are given in Table 1.0. The variation in the composition of the honeys

TABLE 1

Composition of honey from various *Apis* and *Trigona* species

Honeybee		No. of samples	Water %	Glucose	Fructose	Sucrose	Dextrin	Ash %	Protein %	Undetermined %	Total Solids %
Apis mellifera	White *et al.*, (1962)	490	17.20	31.19	38.28	1.31	1.50	0.17	*0.26	3.10	82.80
	Minh *et al.*, (1971)	1	20.66	35.28	29.41	1.27	0.22	0.18	*0.26	8.16	79.34
Apis cerena	Phadke (1967)	80	20.89	33.39	36.48	-	1.97	0.19	0.56	2.18	77.57
	Phadke (1962)	9	18.32	34.19	39.83	-	1.46	0.19	0.64	1.79	79.89
Apis dorsata	Minh *et al.*, (1971)	8	27.81	28.98	30.65	1.50	0.81	0.17	*0.50	4.32	72.19
	Phadke (1968)	20	20.89	32.13	37.43	-	1.57	0.39	1.07	2.14	75.22
Apis florea	Phadke (1968)	5	16.5	32.34	38.94	-	8.66	0.37	1.34	3.19	83.50
Trigona	Phadke (1968)	5	24.05	20.05	32.34	-	5.89	0.52	0.78	3.58	75.50

* These are average protein percentage values calculated by multiplying the original nitrogen value by the factor 6.25.

produced by these species of honeybees arises not only due to their *genii* but also from the environmental conditions. Vorwohl (1968) reported that the amylase activity of the *Apis cerena* honeys were lower than those of the *Apis mellifera* honeys obtained from the same locality. Davies (1975) reported that the honey from *Apis cerena indica* had a low proline content compared to that of the *Apis mellifera*. Klemarewski (1976) observed that the activities of the enzymes amylase and invertase were higher in the honeys produced by the *Apis mellifera mellifera* than those collected by the *Apis mellifera caucasia* and the *Apis mellifera carnica*. Wakhle and Desai (1983) reported that honey produced by *Apis cerena*, *Apis dorsata*, *Apis florea* and *Trigona iridipennis* had a high invertase content but low diastase, catalase and glucose oxidase content in comparison with reported values for *Apis mellifera* honeys. Also *Apis dorsata* honey had the highest content values for diatase, invertase and catalase and *Trigona iridipennis* had the least but displayed highest glucose oxidase activity compared to the others.

The Amino Acid Composition of Nectar and Honeydew

The raw materials for the production of honey by the honeybee are nectar and honeydew. Nectar is secreted by the plant glands situated in the region of the flowers or in other parts of the plant called 'floral' and 'extrafloral' nectaries, respectively. Honeydew is secreted by the plant-sucking insects such as aphids, coccoids, leaf hoppers feeding on the phloem or sap of plants and trees.

The free amino acids of nectar

Mostowska (1965) reported the presence of the following amino acids in the nectars of *Brassica napus var. oleifera*, *Robinia pseudoacacia*, *Tilia cordata*, *Trifolium repens*, *Phacelia tanacetifolia* and *Calluna vulgaris*. These amino acids were: alanine, arginine, aspartic acid, glutamic acid, histidine, leucine, lysine, ornithine, phenylalanine, proline, threonine tyrosine and valine. He found that the amino acid content of nectar ranged from 0.002 to 4.786 mg per 100g of dry matter.

Hanny and Elmore (1974) used the technique of gas- and thin-layer chromatography and identified twenty amino acids from the extra-floral nectar of cotton *(Gossipium hirsutum)*. These amino acids were: alanine, α-amino adipic acid, γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, 2,4-diaminobutyric acid, glutamic acid, glutamine, glycine, isoleucine, leucine, lysine, methionine, ornithine, proline, serine, tyrosine and valine. These researchers noted that the quantitative amino acid composition of the cotton extrafloral nectar fluctuated because of the environmental conditions but the qualitative composition was constant. The percentage nitrogen due to the amino acids constituted on average 0.04% of the extrafloral nectar of cotton.

Baker and Baker (1975) reviewed the constitution of nectar and reported that alanine, aspartic acid, glutamic acid, glycine and serine appeared to be the commonest amino acids of nectar.

Baker, Opler and Baker (1978) reported that the amino acids asparagine, cysteine, lysine and tyrosine were more frequently

represented in the extrafloral nectar whereas these amino acids are only moderately represented in the floral nectar.

Inouye and Inouye (1980) reported the presence of a total of twenty four amino acids in the extrafloral nectar of *Helianthella quinquenervia* more than has been reported for any other extrafloral nectar. These amino acids were: α- and β-alanine, α-amino adipic acid, α- and γ-aminobutyric acid, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, phosphoserine, proline, serine, threonine, tryptophan, tyrosine and valine.

Gilliam, McCaughey and Wintermute (1980) reported that the nectar of citrus cultivars contained from 7.5 to 79.1 mg of amino acids per 100g of nectar and that unlike most plants *Citrus* had low level of proline in its pollen and high level in its nectar. Gilliam, McCaughey and Moffett (1981) compared their results on the composition of amino acids in the floral nectar of cotton *(Gossypium hirsutum)* with those of the extrafloral nectar of cotton as reported by Hanny and Elmore (1974). Gilliam, McCaughey and Moffett reported that the predominant amino acids in the floral nectar were aspartic acid, serine, glutamic acid and threonine; whereas glutamic acid and its amide were dominant in the extrafloral nectar. Thus, confirming the findings of Baker, Opler and Baker (1978) that extrafloral nectar was significantly different in composition from that of floral nectar.

Baker and Baker (1982) analysed the constituents of nectar from over a thousand species of flowering plants and reported that the amino acid composition to have a taxonomic and phylogentic significance

and to be of benefit to both the plants and their pollinators.

Freeman, Reid and Zaun (1983) reported the presence of asparagine, glutamine, phosphoserine, phosphoethanolamine, ornithine and taurine in nectars of four species of *Agave* (Agavaceae). These amino acids have as yet not been reported to be present in nectars. These researchers also found variation in the detection of several amino acids within species.

Pais *et al.*, (1986) reported that the amino acid content of *Limodorum abortivum* (Orchidaceae) and *Epipactis atropurpurea* nectars and the entantiomoric composition of the particular amino acids indicate evolutionary differences of both nectaries.

Bahadur, Chaturvedi and Swamy (1986) examined the nectars of one hundred Indian plant species representing eighty three genera from thirty four families of angiosperms and they reported that eighty five species contained α-amino acids. These research workers observed that the nectar of *Hybanthus ennaespermus* (Violaccae) exhibited a high amino acid content and out of the seven amino acids valine, leucine and glutamic acid were essential for insect nutrition. The nectar of *Spathodea campanulata* (Bignoniaceae) had only two amino acids alanine and isoleucine of which the latter was essential for insect nutrition. The *Turnera subulata* (Turneraceae) nectar was characterised by five to seven amino acids of which leucine, isoleucine, tryptophan and phenylalanine were essential for insect nutrition.

The free amino acids of Honeydew

Mittler (1953) reported that the number and amount of amino acids in honeydew depended on the extent to which these amino acids were present in the phloem sap of the plant. Morgenthaler (1955) has discussed the origins of honeydew and concluded that the raw materials for honeydew were exactly the same as that for nectar. Ewart and Metcalf (1956) have reviewed the literature on amino acids present in honeydew. Mittler (1958) reported that the number and concentration of amino acids and amides in the phloem sap and honeydew fluctuated with the seasonal development of the host plant.

Auclair (1963) has given general references on the composition of honeydew.

Srivastava and Varshney (1966) using the technique of paper partition chromatography identified seventeen free amino acids and amides in the honeydew excreted by *Kerria lacea*. These amino acids were: α- and β-alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tyrosine and valine. These researchers found that the eight amino acids from arginine to tyrosine were absent from the plant sap of *Maghania maerophylla* on which the insects were reared. They suggested that this difference could presumably be due to the action of proteolytic enzymes present in the insect gut. These findings were later confirmed by Salama and Rizk (1969).

Saleh and Salama (1971) reported that the amino acids in the honeydew came directly from the ingested sap and were not the product of either protein breakdown as suggested by Auclair (1963) or

atmospheric nitrogen fixation as suggested by Kennedy and Mittler (1953).

Noda, Sogawa and Saito (1973) found that when insects were reared on distilled water, only traces of amino acids were secreted.

Hertel and Kunkel (1977) noted the variation in the amino acid concentration in the changes of water supply or the phloem sap content which influenced the honeydew of *Myzus persicae*.

Lombard, Rossetti and Buffa (1981) detected seventeen free amino acids and one ninhydrin-reactive substance in the honeydew of *Eucallupterus tiliae* but only fourteen free amino acids and two ninhydrin-reactive substances in the *Tilia cordata* leaf extract. These researchers observed that the honey derived from linden honeydew contained fourteen amino acids and small amounts of proline compared to that of the nectar honey.

Processing of Nectar into Honey by the Honeybee

The way in which nectar is transferred by the forager bee to the housebee and how the housebee is then actively and passively engaged in the processing of nectar into honey has been described in detail by Maurizo (1980a).

The active part played by the housebee involves the concentration of nectar by rapidly pumping out from the honeystomach a thin film of liquid onto the end of its proboscis, thereby exposing the nectar to the warm dry air of the hive for evaporation. When the concentration of the sugars in the nectar reaches approximately 60% (w/v) it is then deposited into the cells of the comb (Park 1933). This is then referred to as unripened honey.

The passive part played by the housebee involves the evaporation of the excess water from this unripened honey in the combs. It takes on average one to three days for the water content of the ripening honey to reach 20% or less. This depends on the level of cells filled, the amount of air movement, temperature and the relative humidity of the hive (Park 1927, 1928, 1933). The housebee then seals the cells with an airtight wax capping to prevent the re-absorption of water from the surrounding air and thus eliminating the risk of fermentation.

It is known that the housebee adds the enzymes diastase, invertase and glucose oxidase (Rinaudo *et al.*, 1973a and 1973b and Bergett 1974), and these will be reviewed elsewhere in the introduction.

Extraction and Processing of Honey

The techniques available for the extraction and processing of the honey to the amateur and the semi-commercial beekeeper in Europe and the United States of America have been described by many authors; for example Morse (1974), Hooper (1976), Smith (1980) and Townsend (1980). The description given by these authors refer to the modern moveable frame hive. Although several types of hives, namely National, W.B.C., Dadant, Langstroth, Smith and Commercial exist which vary somewhat in size, use and regional popularity. All consist of the following components: a set of frames up to ten made of wood or plastic on which honey comb is constructed, brood chamber for queen and colony, extension boxes or supers for the bulk of the combs and honey, crown board, floor and roof. In addition there are sundry traps to control access of bees. Some tropical honeys are gathered

from either wild colonies or from crude straw skips, hollowed logs or clay containers which are smashed and colonies destroyed once harvesting is complete.

The extraction of the honey from the combs involves the removal of the honey combs from the hives, the uncapping of the honey combs; followed by the removal of honey from the uncapped honey combs often with the aid of centrifuges. Then finally the honey is strained to remove the wax bits and extraneous material that may have entered the honey during the extraction process. The normal straining process used by the amateur beekeeper is to warm the honey to 35°C and then strain through a cloth (54 mesh to 1 inch) or a nylon strainer. The semi-commercial beekeeper utilizes high pressure mechanical strainers on a large commercial scale. This process of filtering or polishing with very efficient filter presses can result in the honeys being almost free of pollen (Hicks, 1969).

The processing of the extracted honey involves controlled heating to destroy the yeast present and to dissolve the glucose monohydrate crystals, combined with fine straining or pressure filtration.

Hooper (1976) has described in detail the various methods used for processing extracted honey by the amateur beekeeper. White (1978), Townsend (1980) and Dyce (1980) have given extensive detailed accounts of the processing, packaging and storage of honey for the commercial producer.

Heather honey from ling *(calluna vulgaris)* forms a gelatinous state upon standing due to its unusually high content of a thixotropic protein which imparts this thioxotropic property on it. Hence the

extraction and processing of heather honey is different from that mentioned above. The honey is extracted either by stirring with mechanical probes or needles inserted into cells of the combs before being spun out in the usual way or the honey is pressed out of the combs with the aid of a heather press. The extracted honey can then be heated for two or three days at 40°C before bottling in the usual manner (Hooper, 1976). However, on the other hand combs containing the heather honey can be cut out into convenient sizes and sold as 'cut comb honey' in plastic containers provided there are sufficient commercial outlets to justify the extra care and attention required to ensure perfect combs.

Honey Production and Trade

Estimated figures of the world honey production and trade for the years 1983 and 1984 were obtained from the World Honey Crop Reports No. 21 and 22. The reports list the actual or estimated production from sixty one countries world-wide covering the Americas, Europe, Australasia, Africa and the tropical islands.

Production

In 1983 there was 934,100 metric tons of honey and in 1984 an estimated 940,000 metric tons of honey harvested world-wide.

The estimate of honey production of ten individual countries in 1983 and 1984 have been shown in Table 2.0. The continued trend of a small annual increase in world honey production was reported. This could be attributed to the increase in the number of colonies of the major producers such as the USSR, the improved yield per

TABLE 2

World honey production and trade in 1983 and 1984 in ten selected countries

Country	World honey production*		World honey trade**			
			Exports		Imports	
	1983	1984	1983	1984	1983	1984
Argentina	28.0	33.0	29,874	29,227	-	-
Brazil	22.0	27.0	628	1+	-	-
Canada	34.8	44.1	9,752	9,522	239	284
Mexico	64.0	55.0	40,028	59,405	-	-
U.S.A.	93.0	95.0	3,781	3,384	41,715	49,823
German Fedral Republic	15.0	18.0	12,960	9,409	75,830	66,385
Total USSR	190.0	198.0	16,000	19,890	-	-
Japan	6.5	7.5	-	-	28,146	33,180
China***	100.0	115.0	58,096	64,339	-	-
United Kingdom	1.2	-	-	1,455	20,764	20,743

* - In thousand metric tons.

** - Preliminary estimates in metric tons.

*** - Based on imports of major importing countries.

\+ - This value was quoted in the world honey crop report No. 22 and referred to as a misprint regarding the actual amount of honey exported by Brazil in 1984.

colony in countries such as Canada and Brazil and favourable weather conditions in countries such as Argentina and Brazil. A considerable proportion of the honey produced was for consumption within the country and these countries may not export much surplus honey.

Trading in honey

In 1983 a significant increase in exports of honey was observed in countries such as Russia by 42%, Mexico by 48% and France by 90%. Also, an increase in imports of honey was observed in countries such as the United States of America by 19% and Japan by 18%. Preliminary estimates of honey exports and imports in 1983 and 1984 for ten countries have been shown in Table 2. The largest net importers of honey were the European Economic countries such as the German Federal Republic and the United Kingdom followed by the United States of America and Japan.

Crane (1980) and Willson (1980) have also given figures for the world production and trading in honey.

Manufactured products that use honey, wax or other bee products are numerous and range from mead, cake, candles, cosmetics to health foods. Also there is a wide spread inclusion of honey into pharmaceutical products such as cough syrups. Some honey is also used directly as a therapeutic agent see, for example, Steyn (1973), Willson and Crane (1980). Therein are given examples of minority but still important users of honey and the whole aspect of honey from analysis to archaeology to legend have been covered in the extensive treatise by Crane (1980) and will not be further mentioned.

Honey Plants

Crane (1980) has listed the important plant species which contribute as a source of honey on a world scale. She has also discussed their characteristics and distribution, the characteristics of the honey from them, and their economic significances. Other authors such as White (1978) and Howes (1979) list and discuss the major honey plants in Britain, Europe, United States of America and the rest of the world.

In Britain, honey from clover *(Trifolium sp.)* constitutes about 75% of the total honey produced. While honey from lime *(Tilia sp.)* accounts for the greater part of the remainder. Honey from heather *(Calluna sp.)* in the British Isles is widely recognised as a unique table honey. Oil seed rape *(Brassica sp.)* is fast becoming a major honey crop in Britain (Sims, 1984). Fruit blossom *(Prunus/pyrus)* is another source of nectar and is useful to the beekeeper for brood rearing if not for surplus honey. Apple *(Malus)* and cherry *(Prunus)* are most valuable nectar producers among the fruit trees.

Large quantities of honey from clover are produced by countries such as Canada, Northern United States of America, New Zealand, Australia and Tasmania. Honey from acacia *(Robinia pseudoacacia)* produced in countries such as Hungary, Rumania and Yugoslavia are rated as an equal, in terms of quality, to that of the clover. Buckwheat honey *(Fagopyrum sp.)* is also produced in large quantities in North-eastern United States of America, Russia, China and in parts of Europe. Honey from citrus fruits are well known to Spain and California. Thyme and pine forest honeydew honey from Greece, Also *Eucalyptus*

from Australia and mixed floral honeys from Mexico.

Crane, Walker and Day (1984) have identified four hundred and sixty seven plants to be a major source of honey produced in the world. For each plant these authors have noted the following available information: the botanical and common names and the plant family; the form of plant, its distribution and environmental conditions; economic importance and uses; potential hazards such as toxins; nectar rating and honeybee species in a country; the time of the year of flowering, nectar flow and composition; honey yield and chemical composition; honeydew production; recommendations of planting to increase honey production in a country; and the composition and properties of the honey produced.

Moreover, the sources of honey in the United States of America, Europe, Australia, Canada are well documented compared with the other honey producing countries, although such documentation that exists is kept on file at the International Bee Research Association with the United Kingdom and by national organisations elsewhere.

Honey Composition

In the following section and other sections covering perpherial topics only the major literature reviews of importance to the particular subject will be briefly reviewed. However, for major topics of importance to the present study relevant published research will be described and reviewed in detail.

Factors affecting composition

White (1980c) has reviewed the literature on the composition of honey and suggests that the honey composition is dependent upon the origin of the nectar. Also, that the internal and external factors which influence the production of nectar are important contributory factors to the honey composition.

Maurizo (1980a) has extensively reviewed the literature and discussed in detail how these internal and external factors influence the production of nectar. She has suggested that the size of the flower and the nectary surface, the age and maturity of the flower on the plant, the species, variety or cultivated races to which the plant belongs are some of the internal factors. External factors such as the soil humidity, the type of soil and the use of fertilizers, the temperature and the wind, the time of the day and year, the length of the day and sunshine are also important. Similarly, Shuel (1982) has also outlined the internal and external factors that influence the secretion of nectar.

The affects of beekeeping techniques, extraction and processing of honey either by the beekeeper or by the commercial producer, on the composition of honey have been described by Hooper (1976) and Morse (1974), respectively. Also other factors encountered during the commercial processing have been described by Rodgers (1980) and Townsend (1980). The conditions of storage have a considerable influence on the composition of honey and the changes induced by storage have been described by White, Kushnir and Subers (1964), Echigo, Takenaka and Ichimura (1974) and White (1978).

Physical properties of honey

White (1980d) has extensively reviewed the literature on the physical properties of honey. Honey is a supersaturated solution of carbohydrates in water, with normally only 15% - 17% water.

Moisture content

White *et al.*, (1962) showed that the average moisture content of four hundred and ninety samples of honey was 17.2%. The amount of water present in honey determines its stability against fermentation and granulation (Dyce, 1980). The determination of moisture content has been reviewed extensively by White (1969).

Colour

Brice *et al.*, (1956) showed that the colourants of honey, marple syrup, caramel solution and other sugar products are similar. These researchers have also carried out an extensive physical examination of the colour of honey.

Wootton, Faraji-Haremi and Johnson (1976) reported that when six Australian honeys were stored at 50°C, darkening of honey colour was not accompanied by appreciable changes in the total nitrogen content. Wootton, Edwards and Faraji-Haremi (1976) reported that changes in the amount of sugars and free amino acids in honey were not clearly related to the extent of darkening.

White (1980d) has reviewed the effects of storage temperatures on the colour of honey.

Crystallisation

It is basically the crystallisation of the glucose present in honey to its monohydrate form. The presence or absence of nuclei

influences the onset of crystallisation but its extent and speed are dependent upon the glucose/water ratio (White, 1980d). The major problem which results from crystallisation is the increase in moisture content of the liquid phase above the crystals. This creates ideal conditions for the yeast present to ferment the honey.

The factors affecting crystallisation were studied and reviewed by Dyce (1931). Later on Austin (1958) proposed the use of the [glucose]/water ratio for predicting the crystallisation tendency of honey. It was White *et al.*, (1962) who suggested the use of Austin's ratio as being highly significant as a measure of tendency towards crystallisation. Furthermore, these research workers regarded this ratio as being equivalent to the [glucose]-water/[fructose] ratio employed by Jackson and Silsbee (1924). White *et al.*, (1962) on the basis of Austin's ratio reported that values of 1.7 and lower were generally associated with non-granulation of honeys, whereas values of 2.1 and higher would predictably lead to rapid granulation to a solid. These values were based on the measurements carried out on four hundred and ninty samples of honey they examined.

There are exceptions for non-crystallising honey types such as tupelo, acacia which are low in glucose content and high in fructose. The subjects of crystallisation of honey have been reviewed by Doner (1977), White (1978), White (1980) and Landis and Kevin (1982).

Thixotropic properties

Pryce-Jones (1944) studied the thixotropy in ling (*Calluna sp.*) honey in that the honey mobilised upon stirring from its gelatinous solid state. Pryce-Jones isolated by alcohol precipitation a material

which caused the thixotropic property in heather honey. Further, he demonstrated that by adding this material to clover honey thixotropic properties were imparted to the clover honey. One sample of heather honey was found to contain 1.86% of the protein, whereas other samples contained about 1.5% total protein.

According to White (1978) Pryce-Jones also observed thixotropy in Manuka *(Leptospermium scopatium)* honey of New Zealand. Deodikar *et al.*, (1957) reported similar thixotropy in Karvi *(Carvi calloso)* honey of India. Subsequent project work at Salford over the period 1984 - 1987 has established that the thixotropic agent in heather honey is indeed a protein of molecular weight 50,000. Hamilton (1987).

Chemical composition of honey

Honey contains nearly two hundred compounds which can be arranged into the following major groups: carbohydrates, amino acids, proteins, minerals, vitamins, acids, esters and volatile components, hydroxy-methylfurfural, lipids, and pollen. On rare occasions toxic compounds can be present in low concentrations.

The amino acids, proteins, pollens and toxic substances will be reviewed later on in the introduction.

Carbohydrates

Glucose and fructose constitute about 85% of the honey solids. The presence of sugars such as erlose, isomaltose, maltulose, turanose, nigerose, kijibose, α,β-treholose, gentibiose, laminarebose, sucrose and maltose have been reported by White and Maher (1953), White and Hoban (1959), Watanabe and Aso (1960) and Siddiqui and Furgala (1967).

Siddiqui and Furgala (1968) reported the presence of ten honey trisaccharides, one tetrasaccharide and one pentasaccharide. These researchers also identified melizitose, 3-α-isomaltosylglucose, maltotriose, isomaltotriose, 1-kestose, panose and isomaltopentose. The sugars of honey have been reviewed extensively by Thawley (1969), Siddiqui (1970), Doner (1977), White (1980c) and by Prince (1982).

Minerals

The three aspects of the ash content of honey are: the amount of total ash, the amount of principal constituents and the identities of minor metallic constituents, which occur in extremely minute amounts.

Schuette and his colleagues (1932, 1937, 1938, 1939) reported the presence of mineral elements in honey and suggested a relationship with the degree of pigmentation. This relationship was confirmed by White *et al.*, (1962) in that, honey of light colour classes has a lower mineral content than those of darker honey types. Table 3 summarises the findings of Schuette and his colleagues as presented by White (1978). McLellan (1975) reported that there was generally more potassium in honey than calcium, magnesium or sodium due to the rapidity of potassium secretion by the plant.

Vitamins

The following vitamins have been reported to occur in honey: thiamine, niacin, riboflavin, ascorbic acid, pyridoxine, panthothenic acid, biotin, folic acid and vitamin K. The amount of vitamins vary to a large extent between samples of similar honey and also of different honey types. The occurrence of vitamins in honey have been reviewed by White (1978 and 1980c).

TABLE 3

Mineral content of honey - from the data of Schuette *et al.*, (1932, 1937, 1938, 1939) as given by White (1978)

Mineral elements	Honey colour	Number of samples	Parts per million	
			Range	Average
Potassium(K)	Light	13	100-588	205
	Dark	18	115-4733	1676
Sodium(Na)	Light	13	6-35	18
	Dark	18	9-400	76
Calcium(Ca)	Light	14	23-68	49
	Dark	21	5-266	51
Magnesium(Mg)	Light	14	11-56	19
	Dark	21	7-126	35
Iron(Fe)	Light	10	1.20-4.80	2.40
	Dark	6	0.70-33.50	9.40
Copper(Cu)	Light	10	0.14-0.70	0.29
	Dark	6	0.35-1.04	0.56
Manganese(Mn)	Light	10	0.17-0.44	0.30
	Dark	10	0.46-9.53	4.09
Chlorine(Cl)	Light	10	23-75	52
	Dark	13	48-201	113
Phosphorous(P)	Light	14	23-50	35
	Dark	21	27-58	47
Sulphur(S)	Light	10	36-108	58
	Dark	13	56-126	100
Silica(SiO_2)	Light	10	7-12	9
	Dark	10	5-28	14

Acids

The acids of honey contribute towards its stability against micro-organisms and its flavour. Gluconic acid, the product of an enzyme catalysed reaction of glucose oxidase on glucose of honey, is the predominant acid. The acid composition of honey has been reviewed by Thawley (1969), Stenikraus *et al.*, (1971) and White (1978).

Volatiles

The volatiles of honey which contributes towards its characteristics such as aroma and flavour, has been little studied. However, with the introduction of the technique of gas liquid chromatography a number of volatile components have been identified.

Ten-Hoopen (1963) reported the presence of formaldehyde, acetylaldehyde, acetone, isobutyraldehyde and diacetyl in greater quantities in rape and clover honey than in thyme. Hadorn (1964) reported the presence of methyl anthranilate in lavender honey. Cremer and Riedmann (1965) identified over half of the one hundred and twenty compounds they separated from honey. These researchers reported an increase in pentanol, 2-methyl-1-butanol, 3-methyl-1-butanol and n-propanol, upon storage and suggested these could be the products of ester hydrolysis or may arise from corresponding amino acids. Bicchi, Belliardo and Frattini (1983) analysed multifloral and unifloral honey for compounds responsible for their flavour and identified fifty two compounds. These researchers used the technique of gas liquid and combination of gas liquid chromatography and mass spectroscopy. Bonaga and Giumanini (1986) using the combination of a gas chromatography and a mass spectrometer isolated fifty volatile

components from a unifloral chestnut *(Castanea sativa)* honey. These researchers reported that ninteen of these components had not been previously reported to be present in honey. They suggested that the main component of the mixture (3-aminoacetophenone) may be specific for floral source.

Hydroxymethylfurfural

5-Hydroxymethylfurfural (HMF) results from the acid catalysed dehydration and heat treatment of fructose present in honey.

Schade, Marsh and Eckert (1958) using Winkler's (1955) method for quantifying HMF in honey demonstrated the variability amongst honeys of the effect of storage and heating, and conformational factors influencing HMF formation. White, Kushnir and Subers (1964) showed that fresh honey contains between 0.06 to 0.20 mg of HMF per 100g of honey. White (1978) reported that heat treatment during processing of honey should be limited as excessive amounts of HMF are indicative of the loss of freshness and overheating. White (1980a) reported an average HMF content for one thousand seven hundred and twenty eight samples of commercial honey to be 1.24 mg per 100g of honey. This was based on the data published by Duisberg and Hadron (1966) and Chandler *et al.*, (1974). White (1980a) has reviewed the subject in detail.

Honey Proteins

The nitrogen content of honey is due mainly to the presence of proteins and small amounts of amino acids, and other nitrogen compounds. A considerable portion of honey protein is due to the presence of enzymes. The isolation, characterisation and properties of proteins will now be reviewed.

Proteins

The protein content of honey was initially utilized to distinguish honey from artificial admixtures and blends. The assessment of precipitates of proteinaceous material by tannin solution, phosphotungstic acid or by alcohol, immunological tests or by assignment to solubility groups have been described by White (1978 and 1980c).

Paine *et al.*, (1934) studied the colloidal material separated from honey by the technique of ultrafiltration. Helvey (1953) detected three collodial components present in buckwheat honey by the techniques of ultracentrifugation and moving boundary electrophoresis. Of these three components two were proteins of approximate molecular weights 146,000 and 73,000 and the third was a polysaccharide of molecular weight of about 9,000.

White *et al.*, (1962) reported an average value corresponding to a protein content of 0.25% for four hundred and ninety samples of honey.

White and Kushnir (1967b) separated between four to seven protein fractions by the technique of gel filtration, starch-gel electrophoresis and ion-exchange chromatography. These researchers used the crude protein preparations from eleven floral honeys and honey produced by sugar-feeding to the bees. From these results the above authors deduced that four protein fractions appeared to originate from the bee and two of these had molecular weights of 40,000 and 240,000. While those fractions that originated from the plant were of 98,000 or greater than 400,000 molecular weight.

Bergner and Diemar (1975b) separated the honey proteins into five distinct peaks by chromatography on Sephadex G200 gel. These researchers reported that of these five components, three originated from the bee and were identified to be enzymes invertase, diastase and glucose oxidase. While the two that originated from the plant were not identified.

White and Rudyj (1978) determined the proteins for seven hundred and forty samples of honey using the Lowry photometric analysis. These researchers reported a mean value of 169 mg of protein per 100g of honey.

Bagdanov (1981) used the Bradford/Bio-rad colour reagent method to determine the proteins of eighteen Swiss honeys and sixteen foreign honeys. These researchers reported an average protein content of 129 mg per 100g of honey and noted that there was no significant differences between ten honeydew and twenty four floral honeys.

Croft, Mistry and Washington (1986) were able to resolve as many as eleven protein bands on 10% polyacrylamide gel in the presence of sodium dodecyl sulphate. These researchers were unable to characterise any particular honey sample with regard to its likely geographical origin on the basis of presence or absence of any of these eleven protein bands. They also noted the technique was useful in detection of adulteration and its sensitivity enabled detection of honey that had been obtained from bees fed on pollen substitutes.

Reeder, Richie and Guenther (1986) separated honey proteins by the technique of ultra-thin layer isoelectric focussing and scans for band pattern and intensity were analysed by a chemometrics programme

on a mainframe computer. These researchers reported that the clustering of patterns revealed the ability to distinguish honey based on the geographical location of collection.

Enzymes

The enzymes of honey have up to now been studied predominantly from the view point of quality and destruction of enzymes by heat and other adverse factors. The presence of enzymes such as diastase, invertase, glucose oxidase and catalase in honey have been confirmed. The trivial and systemmatic name of enzymes, type of enzyme activity and kinetic properties together with appropriate references for each of these enzymes have been tabulated in Table 4. Other enzymes such as acid and alkaline phosphatases have been shown to be present in honey but have as yet not been well characterised and therefore will not be reviewed. White (1978) has extensively detailed and reviewed the literature on the enzymes present in honey. White (1980c) has also noted the presence of enzymes such as esterases which have not been studied in detail.

The origins of the enzymes listed above have been identified as being chiefly the honeybee Ammon (1949), Schepartz and Subers (1964), Rinaudo *et al.*, (1973a and 1973b) and Stadelmeier and Bergner (1986). Other sources such as pollen and nectar have been suggested by Vansell and Freeborn (1929), Bergner and Sabir (1977), Giri (1938) and Zalewski (1965).

TABLE 4

The enzymes of honey

Enzyme No. (see key)	Type of activity*	Molecular weight (daltons)	Km (M)	pH	Temp[1] °C	Substrate	Other comments	Refs[2]
3.2.1.1	Hydrolyses α-1,4-glucan links in polysaccharides containing 3 or more α-1,4-linked D-glucose units in a random manner.	21,600		4.7	22-30	polysaccharide starch glycogen		1, 2
				5.3	45-50			
		∿25,000		5.6-5.9	45		Activated in presence of chloride ions.	3 13
3.2.1.2	Hydrolyses α-1,4-glucan linked polysaccharides so as to remove successive maltose units from the non-reducing ends of the chains - produces β-maltose by an inversion.					polysaccharide starch glycogen		
3.2.1.20	α-glucoside + H_2O = an alcohol + D-glucose glucotransferase activity.	51,000		5.9	40-45	sucrose	Fructose is a competitive inhibitor	4, 5
			5.26×10^{-3}	6.0	30	maltose	Synthesis maltotriose from maltose	6
		73,000** 54,000++	4.4×10^{-3}	6.6-6.5	30-35	sucrose	Syntheses 4^G-α, isomaltosylsucrose from sucrose	7
1.1.3.4	β-D-glucose + O_2 = D-glucono-δ-lactone + H_2O_2	below 100,000		6.1			Cyanide ion is a strong inhibitor.	8
			1.55			β-D-glucose	Fructose is both a coupling and an uncompetitive inhibitor, prefers β-D-glucose to α-D-glucose by ratio 6:1, destroyed by photooxidation in visible light region 425-525 nm at pH 3.0.	9, 10 11
1.11.1.6	$H_2O_2 + H_2O_2 = O_2 + 2H_2O$		15.4×10^{-3}	7.0-8.5		hydrogen peroxide		12

N.B. The key to references and other notes relating to this table have been listed on the following page.

Key to references and notes regarding Table 4

Key to references

1. White and Kushnir (1967a)
2. Lampitt, Hughes and Rooke (1930)
3. Rina do *et al.*, (1973b)
4. White and Kushnir (1967a)
5. Rina do *et al.*, (1973a)
6. Takenaka and Echigo (1975)
7. Takenaka and Echigo (1978)
8. Schepa and Subers (1964)
9. Schepartz (1965a)
10. Schepartz (1966a)
11. White and Subers (1964)
12. Schepartz (1966b)
13. Stadelmeier and Bergner (1986)

Notes

Enzyme No.	Systematic Name	Trivial Name
3.2.1.2	α-1,4-glucan 4-glucono-hydrolyase	α-amylase
3.2.1.2	α-1,4-glucan maltohydrolase	β-amylase
3.2.1.20	α-D-glucoside glucohydrolase	α-glucosidase
1.1.3.4	β-D-glucose: O_2 oxido-reductase	Glucose oxidase
1.11.1.6	H_2O_2:H_2O_2 oxido reductase	Catalase

* = Obtained from Commission on Enzymes, 1961.

** = α-glucosidase - I
++ = α-glucosidase - II
} Name allocated to enzyme by researchers Takenaka and Echigo, 1978.

(1) Temps = Temperature

(2) Refs = References

Honey Amino Acids

A relatively small portion of the nitrogen content of honey is ascribed to the free amino acids present in honey. Several workers have attempted to utilize the amino acids of honey in order to determine its authenticity. These workers were: Tillmans and Kiesgen (1927), Schuette and Templin (1930), Schuette and Baldwin (1944) and Vavruch (1952).

Komamine (1960) using the technique of paper chromatography detected the presence of the following twelve amino acids in Finnish and imported honeys. These amino acids were: α- and β-alanine, aspartic acid, cysteine, glutamic acid, glycine, lysine, proline, serine, threonine, tyrosine and valine. Apart from these amino acids the Finnish honey also contained arginine, ethanolamine and methionine and the imported samples contained γ-aminobutyric acid, asparagine and glutamine. He also noted the presence of proline to be the highest in both sets of samples.

Phadke (1962) reported that the dark colour classes of honey produced by *Apis cerena* contained tryptophan and tyrosine whereas the honey graded into the light colour classes did not contain these amino acids.

Kalimi and Sohonie (1964) observed some variation in four Mahabaleshwar (India) honeys, in that, proline, serine and tyrosine were absent in the Gela and Harda honeys. Histidine was absent in both the Gela and Jambul honeys. Isoleucine was not present in any of the honeys.

Petrov (1971) using the technique of ion-exchange and paper chromatography was able to detect the presence of sixteen amino acids in three samples of Australian honeys; stingy bark *(Eucalyptus macrorrhyncha)*, red gum *(Eucalyptus camaldulensis)* and white clover *(Trifolium repens)*. These amino acids were: alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, hydroxyproline, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine. In comparing his results with those of Kalimi and Sohonie (1964) Petrov noted that the Indian honey did not contain cysteine, hydroxyproline, phenylalanine or threonine, whereas isoleucine and methionine were not detected in the Australian honey. In comparing the Australian honey with those of the Italian (Curti and Riganti, 1966) Petrov found that the Italian honey contained most of the amino acids that were present in the Australian honey but not arginine and cysteine.

Bergner and Hahn (1972) reported that using sugar fed bees whilst the amounts of amino acids varied widely, presumably due to the differing content of honeybee secretions, the pattern of amino acids was remarkably similar. The content of the total free amino acids varied from 2,000 to 17,000 nanomoles per g of honey.

Petrov (1974) using an automatic amino acid analyser estimated quantitatively the presence of seventeen amino acids in six *Eucalyptus* and one *Trifolium* honeys. This researcher observed the variation in the amounts of different amino acids and also between different species. He suggested that this variation was due to the amounts of amino acids collected in the nectar, those secreted by the bee

and those formed as degradation products of pollen.

Davies (1975) carried out an extensive survey of the amino acids in ninty eight samples of honey and eight samples of non-honey sugar products. He was the first to suggest that certain ratios of amino acids could be used to determine the geographical source of a honey sample as well as the species of bees. Davies reported that non-honey sugar products could be easily recognised particularly by their lower proline content since honeys generally have a high proline content. Davies also suggested that the bees, nectar and honeydew, were the major sources of amino acids in honey. Later on in 1976, Davies used the amino acid analysis to characterise honey from four geographical areas by a selection of sixty amino acid ratios. He was able to show that while there are variations in the ratios between samples of the same area, the variation between sources is much greater. Thus, Davies proposed that the amino acid analysis could be used as an instrumental method for the determination of the geographical sources of honey, in place of the highly subjective method of pollen analysis. He suggested that further work was required before the method could be recommended for official use.

Wootton, Edwards and Faraji-Haremi (1976) reported that the total free amino acid content decreased during storage of all the honeys they examined, except the honey obtained from the tea tree. These researchers also observed that the amount of some individual amino acids increased in some honeys and they suggested that changes due to protein breakdown more than balanced any loss of amino acids by condensation reactions.

The use of gas chromatography technique to determine the free and protein amino acids of honey were carried out by Bosi and Battaglini (1978). These research workers analysed twenty four nectar and four honeydew honeys and observed differences in the total amino acid content, in the ratio between the amounts of free and protein amino acids, and in the proportions of different amino acids. They concluded that the amino acid spectrum of a honey sample could not be used to characterise honey.

Gilbert *et al.*, (1981) analysed forty five samples of honey obtained from the United Kingdom, Australia, Argentina and Canada. These workers analysed their results statistically using the canonical variates analysis. They were able to show good discrimination between groups of samples from Australia, Argentina and Canada. They observed that the United Kingdom group occurred between the Argentinian and the Canadian groups and could only be discriminated by omitting the Australian group. Gilbert *et al.*, suggested that the free amino acid analysis method must be employed in conjunction with another technique in order to obtain a reliable identification of the country of origin.

Siddiqi (1981) analysed ninty samples of honey using a combination of paper electrophoresis and chromatography. Some samples were also analysed on the automatic amino acid analyser and this technique confirmed the semi-quantitative assessment used for all samples. She was able to demonstrate the presence of pipecolic acid in honey by the technique of mass spectroscopy. Other amino acids grouped as unusual such as citrulline, hydroxypipecolic acid, kynurine

and methyl-histidine were identified from their R_{ARG} values and use of standards. She also demonstrated that a honey sample could be characterised on the basis of its amino acid patterns and their relative proportions obtained following paper electrophoresis and chromatography. Emphasis was placed on the importance of paper electrophoresis and chromatography methods owing to the fact that it not only provided a characteristic pattern but also revealed the presence of trace amounts of amino acids not detected by the automatic amino acid analyser. Furthermore, her technique could be applied in the field by scientifically trained beekeepers with little capital outlay.

Kanematsu *et al.*, (1982) analysed one hundred and twelve honey samples and was able to demonstrate that by plotting ratios of amino acids against one another, honeys from various origins could be distinguished. These research workers showed that by plotting proline/aspartic acid ratio against the total amino acid content the clover and acacia honeys could be distinguished. Also the Chinese milk vetch *(Astergalus sinicus)* honeys originating from Japan and China were distinguished by plotting the isoleucine/leucine ratio against proline/phenylalanine ratio. By plotting the isoleucine/leucine ratio against the proline/glutamic acid ratio acacia honeys from Japan, China, Rumania and Hungary were differentiated.

Davies and Harris (1982) demonstrated that by use of complex statistical analysis of amino acids that it was possible to correctly classify between British and Foreign honeys. These researchers found that the results of identity testing by the ratio method was poor compared to the success of the canonical variates analysis

for the twenty five samples they used. They suggested that the results could not be used more widely without having collected further data to test for the variation due to factors such as season, climate, sampling and laboratory.

Palma, Fontanarrosa and Vigil (1982) reported that their findings conformed with those observed by Petrov (1971). These researchers found that the Indian honeys did not contain cysteine, hydroxyproline, phenylalanine and threonine but contained isoleucine and methionine which were not present in the Australian honeys. They also noted that the Japanese honeys contained methionine and tryptophan in addition to the sixteen amino acids they identified in the Australian honeys.

El-Sayed (1982) reported that the clover honeys examined were representative, and that these samples had a relatively high content of aspartic acid and glutamic acid. This author suggested that certain amino acid ratios could be used for identification of country of origin.

Carter (1983) studied the variation in the free amino acid composition of honey from a single hive during the summer season of 1982. This researcher reported that honey from a mixed floral source is different in terms of its amino acid composition than that of a unifloral source namely *Epilobium augustifolium*. He also noted the variation in the amino acid content during the season. Carter also made calculations to the effect that the contributions of individual amino acids made by the pollens in a jar of honey are negligible in respect to its overall amino acid composition. However, considering a mass of 1 to 2 Kg of pollen which is usually

present in a hive could most certainly influence the amino acid composition of the final honey produced.

Poncini, Wimmer and Vakamoce (1983) reported the presence of sixteen of the twenty seven common amino acids in varying amounts of Fiji honeys. They also noted that leucine, tyrosine, valine, threonine and glycine were found in highest amounts, and cystine and cysteine in lowest amounts.

Chepurnoi (1983) reported that the main amino acids present in the following monofloral honeys were: alanine, asparagine, glutamic acid, glutamine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. These monofloral honeys were: lime *(Tilia sp.)*, sanifoin *(Onobrychis viciifolia)*, *Robinia pseudoacacia*, sunflower *(Helianthus annuus)*, buckwheat *(Fagopyrum sp.)* and *Phacelia tanacetifolia*. Chepurnoi observed that threonine was an important free amino acid of light colour class honeys compared to those of the dark colour class honeys, e.g. buckwheat and Phacelia. In contrast proline was more evident in dark colour class honeys than in the light colour class honeys.

Campus, Madau and Solinas (1983) examined twenty six Sardinian honeys and reported that on average these honeys contained 73.42 mg of amino acids per 100g of dry matter. They also observed that proline constituted on average 69% of the total amino acids and that cysteine, methionine and tryptophan were present in very small amounts.

Bonafaccia *et al.*, (1984) reported that commercial honey samples of dubious origin contained less total free amino acid and less

proline than the genuine honey samples although the free amino acid composition was similar.

Barnard (1986) using the technique of high speed liquid chromatography and fluorescence detection was able to separate o-phthaladehyde derivatives of amino acids in honey. This researcher reported that sixteen amino acids were detected within twenty minutes of sample application, however, the presence of proline, hydroxyproline, cysteine and cystine could not be determined. This was because o-phthaldehyde does not react with the imino acids and gave low fluorescence yields with cysteine and cystine.

Speer and Montag (1986) examined forty five individual honey samples from the following sources for the presence of free amino acids using the technique of ion-exchange chromatography. The sources were: Chestnut, acacia, rape, dandelion, clover, Tasmanian leatherwood and heath. These research workers reported that heath honey had the highest level of free amino acids. They also reported that amino acid ratios, proline/phenylalanine and proline/tyrosine, could be used to distinguish French and German heath honeys.

Honey Pollens

Pollen is the chief source of proteins for both the larval and adult bees. Without the proteins the honeybee cannot develop and grow (Howells, 1969). The collection of pollen by the honeybee has been discussed in detail by Free (1970) and Butler (1972).

The chemical composition of pollen obtained from different kinds of flowers varies enormously. Analysis of pollen from thirty two different plant species carried out by Todd and Bretherick (1942)

revealed that pollen contained 21% proteins, 11% water, 30% carbohydrates, about 5% fats, oils and waxes and mineral elements such as potassium, phosphorous, calcium, magnesium and iron were also shown to be present. The presence of other constituents such as amino acids, organic acids, sterols, nucleic acids and pigments, and those listed above have been reviewed in detail by Stanley and Linskens (1974). Crane, Walker and Day (1984) have listed the colour, grain, yield and chemical composition of pollens for four hundred and sixty seven plants which are a major source of honey produced in the world. However, only the amino acid composition of pollens will be considered here in detail.

The Free Amino Acids of Pollens

Nielsen, Grommer and Lunden (1955) reported the presence of α-aminobutyric acid and hydroxyproline in *Zea mays* pollen. Virtanen and Kari (1955) noted the presence of hydroxyproline and pipecolic acid in the pollen of six wind pollinated plants.

Britikov and Musatova (1964) reported an extraordinarily high amount of proline in the pollen from sixty four plant families they examined. Shellard and Jolliffe (1968) reported that there was no species difference in the amino acid content of eleven grass pollens which they examined.

Lotti and Anelli (1972) using an automatic amino acid analyser determined the free amino acid composition of twenty hand harvested pollens belonging to fourteen botanical families. These research workers reported that the free amino acid nitrogen represented a high fraction, up to 95% of soluble nitrogen content of the pollens.

Gilliam, McCaughey and Wintermute (1980) reported that aspartic acid and glutamic acid were the predominant amino acids in the pollens from citrus cultivar flowers. These researchers also noted that stored pollen contained higher amounts of proline than the flower pollen.

Kauffeld (1980) showed that the following ninteen amino acids were present in the pollen collected by *Apis mellifera*. These amino acids were: α- and β-alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. This researcher noted that the amino acids glycine, lysine, phenylalanine and proline showed considerable variation throughout a year.

Ceausescu and Mosarie (1981) identified ten amino acids by the technique of paper chromatography in the monofloral pollens collected by *Apis mellifera carpatica*. These research workers reported that Compositae pollen contained alanine, aminobutyric acid, arginine, glutamic acid, hydroxyproline, lysine, methionine, norvaline, proline and serine; Ranunclaceae pollen contained alanine, aminobutyric acid, glutamine, glycine, hydroxyproline, leucine, lysine, phenylalanine, proline and valine; and Jaglandaceae pollen contained arginine, cysteine, hydroxyproline, leucine, methionine, proline, serine, threonine, tryptophan and tyrosine.

Zhu and Jiang (1982) observed that the free amino acid content of pollen showed species variation, in that, pollen of *Pinus elliottii* contained arginine and valine whereas pollen of *Ginko biloba* contained serine and tyrosine.

Nadezhdin *et al.*, (1983) reported that the free amino acid constituted approximately 43% of the total pollen amino acid. These researchers noted that the Siberian larch pollen contained more free amino acids than the pollen of the other conifers.

Baruch and Sharma (1984) observed wide variation in the number and type of amino acids in the pollen from ninteen plants they examined.

Naumkin (1984) showed the presence of alanine, arginine, asparagine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine in the pollen of the following plants. These plants were: buckwheat *(Fagopyrum sp.)*, raddish *(Raphanus sativus)*, cornflower *(Centaura sp.)*, red clover *(Trifolium pratense)*, sow-thistle *(Sonchus sp.)*, codlins-and-cream *(Epilobium hirsutum)*, burdock *(Archium sp.)* and musk-thistle *(Carduus sp.)*. This researcher reported that raddish pollen was found to be the richest in amino acids whereas the buckwheat and musk-thistle were found to be the poorest.

Wang *et al.*, (1985) found that the free amino acid content of linden pollen was high compared to that of rape pollen.

Feo *et al.*, (1985) reported that proline was found in large amounts in both the pollens of *Cunninghamia lanceolata* and *Cephalotaxus drupacea* and β-alanine was found in trace amounts in the former pollen.

Rayner and Langridge (1985) examined the amino acid content of bee-collected pollen from ten indigenous and sixteen exotic Australian plants. These researchers noted that the amino acid pattern for pollen from both these plant sources indicated that,

generally, the level of amino acids in the pollens were above bee requirements. They also reported that the amino acid tryptophan whose low concentration had been found to be rate limiting for the honeybee!s nutrition.

Mellissopalynology

The way in which pollen grains and other microscopical particles such as root, dust, yeast, spores and other constituents enter the honey have been discussed by Maurizo (1980). It is the microscopical analysis of these elements found in honey which is known as 'mellissopalynology'. Typical authorities on the practice of mellissopalynology are Louveaux, Maurizo and Vorwohl (1970 and 1978) and Sawyer (1975).

This microscopical analysis of honey not only reveals the botanical and geographical origin but also the presence of honeydew, adulteration and the extraction techniques. This was demonstrated by Louveaux, Maurizo and Vorwohl (1970 and 1978).

Much of the development of the pollen analysis techniques had been carried out in Europe during the pre-war period 1930 - 1940. The International Commission for Bee Botany (1953) published a method for pollen analysis of honey and this method was revised and reviewed in 1962. Louveaux, Maurizo and Vorwohl (1970) established the present method of melissopalynology and this technique was later (1978) up-dated to include the 'acetolysis' method recommended for use on honey. Briefly, the procedure involved the addition of a mixture containing sulphuric acid (1 ml) and acetic anhydride (9 ml) to the sediment obtained after centrifugation (2,500 revolutions

per minute) of honey diluted with water (w/v 3:5) to remove carbohydrates, and the resulting mixture was then incubated at 70°C, centifuged and the supernatant removed. This sediment was re-suspended in a mixture of distilled water and detergent, and this supernatant was decanted and the final sediment was mixed with a medium containing glycerine/gelatin prior to mounting. There are many variations in the procedure of acetolysis chiefly to accommodate various aspects of palynological investigations such as fossils, archaeology, pollen allergy and forensic science. Usually these acetolysis procedures are preceeded or in some cases succeeded by a clarification step. This step involves the removal of extraneous matter, complex acidic components present in soil or cellulose or lignin from plants structures (Faegri and Iverson, 1975).

In the first extensive survey of honey produced in the British Isles, Deans (1957) carried out pollen analysis on eight hundred and fifty four samples of honey in order to establish the source of honey. This author recorded the presence of sixty six species of pollen in honey samples he examined. He reported that the most wide spread important honey sources in the British Isles in 1952 were the White clover *(Trifolium repens)*, fruit *(Prunus/pyrus)*, raspberry/blackberry *(Rubus)* and heather *(Calluna)*.

Chaubal and Deodikar (1965) carried out an extensive morphological characterisation of pollen grains of some major, minor and accessory bee plants from the western regions of India. These researchers also determined the seasonal variation in the pollen composition of honey during the successive honey flows in the annual cycle. An extensive list of the pollen grains which were identified has

also been presented.

Howells (1969) examined the pollens from seventy two Welsh honeys and reported that the *Trifolium repens* occurred as a major source of pollen in 64% of the samples followed by *Brassica* pollen in 18% of the samples. While *Prunus/pyrus* ranked third with 15% of the samples. However, *Prunus/pyrus* also ranked as the most important secondary source with 48% of the samples.

James (1969) reported that the classification of honey pollens was not difficult at the plant family level. However, an exact identification at species level was more demanding and complicated further by the commercial practice of filtering honey.

Lieux (1972) identified fifty eight different pollen types in fifty four commercial Louisiana honeys. This author reported that the honeys collected from different regions of Louisiana were not easily distinguished from one another geographically by pollen types, except in a few instances. The major honey plants from this region were of the Fabaceae family, predominantly *Trifolium repens*, *Rubus*, *Berchemia scandens* and *Salix*.

Sawyer (1975) analysed the pollen grains of one hundred and twenty eight samples of imported honey in England. He described the major common pollen grains that are characteristic of the individual countries.

Adams, Smith and Townsend (1979) carried out pollen analysis of samples of nectar shaken from the combs at frequent intervals throughout a season. These researchers identified thirteen taxa in the honey samples collected at the end of the season, whereas

a cumalative total of sixty two were found in the nectar samples. In this way they were able to obtain a much more complete picture of the nectar sources used by a colony over its active season.

Adams and Smith (1981) suggested that a study of the variations of pollen pattern with season of a given nectar source and of the associated honey derived therefrom would be beneficial. The lack of such information at present indicated the need for further investigations to justify the reliability of pollen analysis as a sole indicator of floral source.

Sawyer (1981) has described a relatively simple method of microscopical identification of pollen grains present in honey using key index cards and microphotographs of two hundred and fifty four pollen grains. He has also given an identification key for the pollen grains of one hundred and thirty four plant species, describing their size, shape, aperture number and type, surface and structural features and the exine section.

Weber (1982) used a light microscope and also a scanning electron microscope in order to identify the presence of tricolporate pollen grains belonging to the Fagaleae, *Quercus* group in eleven orange honeys from the East coast of Spain. Only three honey samples out of the eleven were considered as orange honeys whereas the other eight samples contained more *Quercus ilex* pollen than the *citrus*. Weber concluded that because of the sweet smell of orange blossom these samples contained large quantities of citrus nectar.

Porras (1982) studied the pollen spectrum of the sediments of one hundred samples of honey obtained from the Sierra of Aracena, Spain. This researcher reported that the most characteristic botanical species of honey survey belonged to the *Eucalyptus*, *Cistus*, *Erica* and *Echium* pollen types. The honeys examined were of nectar origin as evaluated by their pollen content.

Varis, Helnuis and Koivulehta (1982) analysed the pollen spectrum of one hundred and sixty samples representing the whole beekeeping area of Finland in the years 1977 and 1978. The Brassicaceae pollen grains were the most frequent and abundant type. These pollen grains were: *Trifolium repens* and *Trifolium hybridium*, *salix* spp.; The Rosaceae group that is, *Prunus*, *Malus*, *Sorbus* spp.; *Apiaceae* and *Filipendula ulmaria*. These researchers were able to explain the seasonal variation in spectrum caused by weather conditions.

Feller-Demalsy (1983) examined one hundred and sixty four samples of honey from Quebec, Canada, which had been collected in 1977 and 1978. This author reported that the pollen species most frequently represented in these honeys were: *Trifolium (hybridium* and/or *repens)*, *Vicia cracca*, *Rubus sp.*, *Trifolium pratense*, *Solidago*, *Salix sp.* and *Melilotus sp.*

Moar (1985) carried out pollen analysis on one hundred and ninteen samples of New Zealand honey. He reported that *Trifolium repens* (White clover) is the most important nectar source available to bees in New Zealand and its pollen occurred as a dominant, secondary and minor type. This researcher found that there were

generally more pollen-types derived from wind pollinated plants in honeydew honey than in floral honey. Also noted was that wind pollinated plants such as grass, plantain and *Coprosma* were worked by bees for pollen. Moar observed that honey originating from a particular district of New Zealand could be determined within limits of pollen analysis but such deductions were dependent upon careful pollen identification together with a sound background knowledge of flora and vegetation of New Zealand.

The Characteristics of Pollens in Honey

Pollen grains from different plants vary considerably in their size, shape and structure. These variations have to be taken into account during pollen analysis. The smallest pollen grains that are found in honey are those of the *Myosotis arvensis* (the common forget-me-not). These grains are only 6 µm in size. The largest are the *Abies* (Fir) occurring up to 145 µm in size, (Sawyer, 1981).

However, if the same number of two different pollen grains are to be found in a honey sample this does not mean that the same amount of nectar from the two plants was collected. Moreover, due to their size the small pollen grains of *Myosotis arvensis*, *Castanea sativa*, *Cynoglossum officinale* and *Mimosa pudia* are known to be 'over-represented' in honeys. Whereas, on the other hand 'under-representation' may be due to the bees deliberately dusting off the pollen after collecting the nectar, those of *Tilia* (Lime) pollen (Howells, 1969). In some citrus varieties the anthers are almost sterile and hence only ten to twenty percent of the

citrus pollen may be present in a honey sample. The pollens of *Robinia pseudoacacia*, *chamaencrion*, *Banksia*, Curcurbitaceae, *Eplilobium* and *Rosmarinus* are known to be under-represented (Louveaux, Maurizo and Vorwohl, 1970 and 1978). The pollens from thyme *(Thymus vulgaris*, *citrus* and rewarewa *(Knightia)* were under-represented in New Zealand honey. Whereas pollen from manuka *(Liptospermum)* and kamahi *(Weinmannia)* were over-represented in New Zealand honey (Moar, 1985).

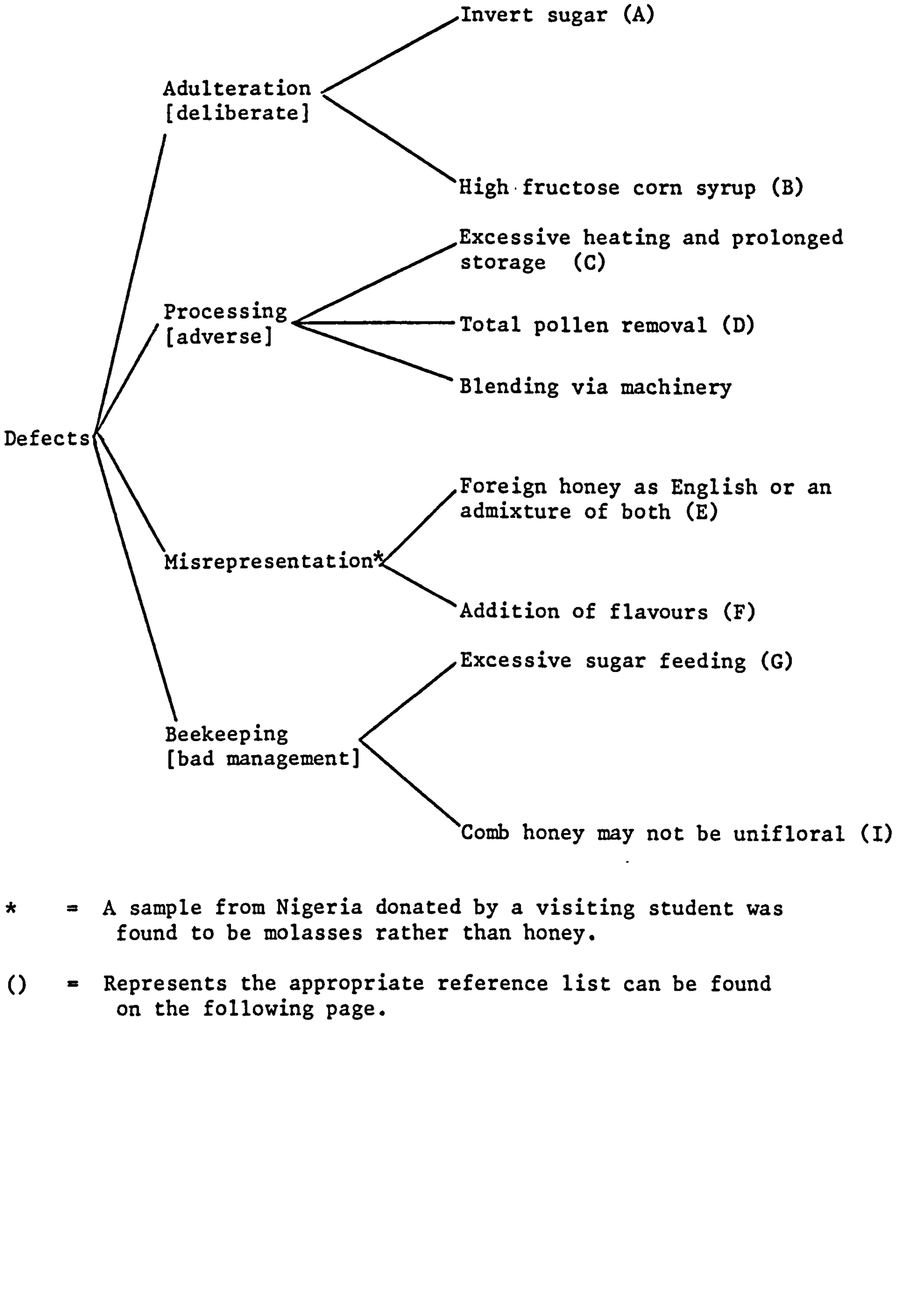
Honey Adulteration

Honey being essentially a flavoured sugar solution now much higher in price than other sweetening agents leads to the temptation of possible adulteration. Under this broad heading one can group misrepresentation, adverse processing conditions, bad practice by the beekeeper and finally delibrate addition of water, glucose or fructose syrups and especially of high fructose syrup, or of artificial essences. It is important to distinguish these adulterated products sold as honey from the artificial honeys deliberately made and sold as such to provide a 'honey flavour' for the catering and confectionary trades. The various aspects associated with each of the four defects have been shown in Figure 1. Reports found in the literature pertaining to the various aspects of defects in the production of honey have also been shown. Usually these practices lead to the lowering of the quality of honey.

Various techniques have been developed and utilized to determine the extent and degree of these practices. At first the technique developed were aimed at the addition of acid inverted syrups. The

FIGURE 1

Defects in the production of honey



* = A sample from Nigeria donated by a visiting student was found to be molasses rather than honey.

() = Represents the appropriate reference list can be found on the following page.

List of references for Figure 1

A = Wiley (1892)
Shannon (1916)
Sherwood (1924)

B = Doner and White (1977)
White and Doner (1978)
Doner, White and Philips (1979)
Kushnir (1979)
White (1979)
White and Robinson (1983)
Kanematsu (1983)

C = Schade, Marsh and Eckert (1958)
White, Kushnir and Subers (1964)
Hase *et al.*, (1973)
White (1980a)
White and Siciliano (1980)

D = Hicks (1969) general commercial practice for many products so as to enhance sales appeal. In the case of honey removes possibility of identification by pollen analysis.

E = Siddiqi (1981)
Sawyer (1975)

F = Croft (1980)

G = Siddiqi (1981)

resorcinol (Fiche) Test (1908) and aniline (Feder) test (1911) have since then been intensively studied and modified. The measurements of the levels of hydroxymethylfurfural as an indication of addition of invert syrup have proven to be inadequate. This was due to the fact that the hydroxymethylfurfural levels increase during prolonged storage at high ambient temperatures or that the honey has been subjected to excessive heating (Schade, Marsh and Eckert, 1958, White, Kushnir and Subers, 1964 and White, 1978). The measurement of the amount of sediment obtained during the extraction of honey for the purpose of pollen analysis have been reported by Louveaux, Maurizo and Vorwohl (1970). These researchers suggested that low amounts of sediment indicate that either the honey was naturally poor in pollen or that the honey had been excessively filtered, or sugar-fed. The apicultural and botanical factors that are involved in the detection of misrepresentation of honey have been reported by Sawyer (1975). Maurikos *et al.*, (1978) reported inconsistent patterns of free amino acids during two dimensional paper or thin layer chromatography in honey adulterated with invert sugar.

High fructose corn syrup (HFCS) has become a major source of possible honey adulterant. This is because HFCS can be produced relatviely cheaply and in bulk by using immobilised glucose isomerase enzymes (Mermelstein, 1975). Instrumental methods have been examined and developed to determine the presence of HFCS in adulterated honeys. A method has become predominant in the detection of HFCS in honey. This method utilizes a highly specialised isotope ratio mass spectrometer to detect the presence of stable carbon isotopes of atomic mass 12 and 13 (Horwitz, 1980).

In order to explain that there is a difference in utilization of isotopes of carbon 12 and 13 by plants which can be measured, although very expensively in a mass spectrophotometer, it is necessary to briefly review current biochemical theory.

It is known that the major sources of nectar of the honeybee are the three-carbon plants (C_3) and the high fructose corn syrup obtained from corn (maize) is known to be a four-carbon plant (C_4), using the terminology as used by Edwards and Walker (1983). The differences in the C_3 and C_4 plants is in the assimilation of atmospheric carbon dioxide.

In C_3 plants the atmospheric carbon dioxide is taken up by the Bassham-Benson-Calvin cycle. Here the CO_2 is first bound to ribulose-1,5-diphosphate by the enzyme ribulose-1,5-diphosphate carboxylase present in the chloroplasts. The resulting six-carbon compound is immediately converted to two molecules of the three-carbon compound, 3-phosphoglycerate (Prince, 1983).

In C_4 plants the atmospheric carbon dioxide is taken up by the Kartschak-Hatch-Slack cycle. In this case the CO_2 is first bound to phosphoenolpyruvate by the enzyme phosphoenolpyruvate carboxylase present in the mesophyll cells. The immediate product is the four-carbon compound oxaloacetate. This compound oxaloacetate is then transferred from the mesophyll cells to the bundle sheath cells and thence to the chloroplasts therein. A decarboxylating enzyme releases the carbon dioxide molecule bound to form the oxaloacetate, to reform phosphoenolpyruvate The released carbon dioxide then enters the Bassham-Benson-Calvin cycle as described above (Prince, 1983).

This difference in the metabolism between the C_3 and C_4 plants results in the phenomenon known as isotope enrichment. Since atmospheric carbon dioxide contains approximately 1% $^{13}CO_2$ and the remainder is $^{12}CO_2$. The differences in the isotope abundance is expressed by the isotope ratio $\delta^{13}C$ expressed as parts per thousand ($^{o}/oo$) (Edwards and Walker, 1983). It is known that the enzyme ribulose-1,5-diphosphate carboxylase discriminates against $^{13}CO_2$ more than the enzyme phosphoenolpyruvate carboxylase. The net effect is that the C_3 plants produce sugar with a $\delta^{13}C$ value of $-28.3^{o}/oo$ whereas the C_4 plants produce sugar with a $\delta^{13}C$ value of $-9.0^{o}/oo$ (Prince, 1983 and Croft, 1987).

White and Doner (1978) showed that honey has a $\delta^{13}C$ value of $-25.4^{o}/oo$ with a range of -22.5 to $-27.4^{o}/oo$ and high fructose corn syrup has a $\delta^{13}C$ value of $-9.7^{o}/oo$ with a range of -9.5 to $-9.8^{o}/oo$. Hence the carbon isotope method can be used to detect honey adulterated with high fructose corn syrup. Invert sugar produced from sugar cane can also be distinguished since it has a $\delta^{13}C$ value of $-11.0^{o}/oo$ (Edwards and Walker, 1983).

Other methods have also been developed to detect honey adulterated with high fructose corn syrup. These are: the thin layer chromatography on silica gel (Kushnir, 1979) and high pressure liquid chromatography (Kanematsu *et al.*, 1983).

Serra and Gomez (1986) applied four different methods to detect adulteration of honey with four different sugar products. These methods and sugar products were: the determination of hydroxy-methylfurfural enabled the detection of invert sugar adulterant at a level of 5% or more; the adulterant common glucose syrup at a

in honey from New Zealand have been extensively studied by Hodges and White (1966), Turner and Clinch (1968) and Clinch and Turner (1968 and 1975). Palmer-Jones (1968) reported that nectar from the Karaka tree *(Corynocarpus caevigata)* was only poisonous to the adult worker bees.

Scott *et al.*, (1971) reported the presence of graynotoxin II and III in a honey sample from the region of Grouse Mountain in the province of British Colombia in Canada. These two compounds are closely related to graynotoxin I (andromedotoxin) which was not found in this honey sample.

White (1973) has extensively reviewed the literature on toxic honey and has listed the reported symptoms after the consumption of poisonous honeys by animals and humans.

Jachimowicz and El-sherbiny (1975) reported that invert sugar containing 30 mg of hydroxymethylfurfural per 100g of honey was toxic when fed to the honeybee.

Majak, Neufeld and Corner (1980) reported that the nectar of *Astragalus miser v. serotinus* was found to contain a misertoxin, a nitropropanol glycocide which resulted in the poisoning of the honeybees.

White (1981) has reviewed the literature on the occurrence and identification of the toxic compounds in honey. This author has described the symptoms of poisoning by these compounds in experimental animals and human subjects. The structure of six of these toxic compounds, that is, acetylandromedol, andromedol, anhydroandromedol, gelsemine, tutin and hyenanchin have also been given.

Atkins (1982) has discussed in detail the effects of poisoning of the honeybees by pesticides and has also listed the plants which are poisonous to them. These plants are: the california buckeye *(Aesculus californica)*, black night shade *(Solanum nigrum)*, death carnas *(Zygadenus venenosus)*, dodder *(Cuscuta sp.)*, summer titi or leather-wood *(Cyrilla racimiflora)*, locoweeds *(Astragalus sp.)*, mountain laurel *(Kalmia latifolia)*, seaside arrowgrass *(Triglochin maritima)*, whorled milkweed *(Asclepias subnerticillata)*, western false hellebore *(Veretum californicum)*, hellebore *(Veretum album)*, henbane *(Hyoscyamus niger)*, horse chestnut *(Aesculus hippocastanum)* and rhododendrons *(Rhododendron sp.)* and its hybrids.

With the increased use of pesticides and insecticides to deter insects that cause damage and/or destruction of crops are also liable to have similar effects on the honeybee. The spraying of crops with these insecticides or pesticides must be carried out so that the mortality rate of the individual honeybees or the honeybee colony is negligible or minimised. The effects of insecticides on the honeybee have been studied by Arzone (1984) and Homan and Baird (1983).

Apart from the presence of toxic compounds in low concentrations, honey is also subject to unpleasant flavours and aroma or bitter tasting. The main origins of these characteristics are due to source of nectar collected by the honeybees. In the British Isles honey from ragwort *(Senecio sp.)* has been described by Howes (1979) to have a strong flavour and aroma which is somewhat nauseous. Also, honey from privet *(Ligustrum sp.)* has been described to have a distinctly bitter taste and was quoted to be quite uneatable.

Further, it was pointed out by Hooper (1976) that these offensive flavours and aroma are often lost when crystallisation and processing are completed. Howes (1979) also noted that honeydew from lime has been reported to have caused fatalities amongst honeybees working limes such as *Tilia petiolaris*, *Tilia tomentosa* and *Tilia orbicularis* flowering late in the season. In other parts of the world, Europe, Americas, Australia, honeys from *Agave*, *Helianthus annuus*, *Senecio jacobaea* and *Castanea sativa* plant species have been reported to have unpleasant flavours, strong aroma and possess a bitter taste (Crane, 1980) and other sources have been listed by Crane, Walker and Day (1984).

AIMS

1. To determine selected chemical components of honey and to show the variation of such compounds with floral or geographical source.
2. The determination of the free amino acids of honey and the use of this information to aid identification as to source even in highly strained pollen-free samples.
3. The correlation of chemical methods with the traditional pollen analysis and with computer predictions.
4. To explore the possible uses of chemical identification to detect adulteration, misrepresentation and gross mishandling of honey.

E X P E R I M E N T A L

CHAPTER 2

E X P E R I M E N T A L

CHAPTER 2

Equipment

Amicon Standard cell No. 402, filtration unit and a UM10 membrane filter to exclude material below 10,000 daltons molecular weight were purchased from Amicon Ltd., High Wycombe, Bucks.

Electrophoresis tank, PAGE and 'tent' tanks, 'Shandon' chromatank and the 'Shandon' vokam power pack, 400V output were purchased from Shandon Southern Instruments Ltd., Carnerley, Surrey.

Olympus FHT microscope fitted with triocular head, eyepiece X10 and objectives X10 and X40, were purchased through Gallenkamp (London).

Olympus OM2 camera was fitted with microscope adaptors and was used to record pollen grains at the recommended magnification of X400.

Technicon TSM1 Multisampler amino acid analyser was purchased from Technicon Instruments Ltd., Hailton Close, Basingstoke, Hants.

Gel scanner Model UA-5 was purchased from Instrumentation Specialities Company, Lincoln, Nebraska, U.S.A.

SP8-500 UV/VIS Spectrophotometer PYE Unicam was purchased from PYE UNICAM Ltd., York Street, Cambridge.

Plastic bottles (100 mls) were purchased from Kartell Plastics (UK) Ltd., Unit 2, Broad Lane, Cottenham, Cambridge.

Materials

Normal laboratory chemicals of analytical reagent quality and distilled water were used as required. Given below is a partial listing of chemicals used. These were:

Bovine serum albumin, Invertase grade V free of catalase and α-galactosidase-activity, 30 - 50 units per mg of solid, Bakers yeast and Folin Ciocalteau's reagent were purchased from Sigma Chemical Company, London.

Acrylamide, N,N'-methyl-bis-acrylamide, sodium dodecyl sulphate, Coomassie Brilliant Blue and Dowex resin 50-X8, 20 - 50 μs Mech (Na) were purchased from BDH Chemicals Ltd., Poole.

N,N',N",N"'-tetramethylenediamine and β-mercaptoethanol were purchased from Koch Light Laboratory Ltd., Colnbrook, Buckinghamshire.

Napthalene Black 12B, propan-2-ol analar grade and Basic fushin were purchased from Hopkin and Williams, Chadwell, Health, Essex.

Most of the honeys received were samples provided through the courtesy and arrangements made by the British Beekeepers Association who also made a donation towards the cost of the survey. These survey samples were of known origin and provenance.

Many of the honeys of foreign origin were samples taken from the bulk containers as shipped and provided by Manley Ratcliffe Ltd., Berinsfield, Oxford. Other 1 lb jars of various honeys were either donated by named individuals or by retail purchase.

All the honey samples received were catalogued and listed in Appendix I, according to the label or information provided.

METHODS

Preparation of Honey Samples for Analysis

All the honey samples apart from the China Buckwheat, Code No. 209, which was stored at 4°C, were stored at room temperature, 18 - 22°C. Honey samples that were received in pieces of combs or containing comb

fragments and wax were first strained before any analysis could be performed on them. Also those samples which were soft-set or creamed required thorough stirring. Those samples that had crystallised or were solid upon receiving required gentle warming at 40°C with stirring to dissolve the crystals.

Preparation of the China buckwheat Honey (Code No. 209)

The China buckwheat honey when received in a fifty pound plastic container contained bees and bee fragments, comb fragments and wax, and above all most of it had crystallised. This plastic container was placed in an oven maintained at 45°C, for twenty four hours. This honey was then strained through a layer of mesh and bench cloth. The debris on the sieve cloth and the remaining crystallised honey was warmed at 55°C and stirring thoroughly to break up the crystals. This semi-solid honey was left at this temperature, overnight. Before straining the honey was again stirred to ensure that all the crystalline honey had dissolved. The remaining debri on the sieve cloth was discarded. Both these portions of strained China buckwheat honey were pooled and collected in ten plastic containers (500 mls), and the remainder in its original cleaned plastic container. This honey was then stored at 4°C until required.

Preparation of the United Kingdom survey honeys

These honey samples were received in plastic containers (100 mls). Those samples that were received within pieces of combs or with fragments of combs and wax were first strained before being analysed. These samples were strained through a mesh cloth placed over the mouth of a beaker (100 mls) and then left in an oven maintained at 40°C for an hour.

Crushing the combs with the aid of a glass rod often facilitated the straining process. Occassionally it was necessary to stir the crushed comb and honey on the mesh cloth. The comb fragments with the residue honey that remained on it were discarded. This strained honey was then collected in a clean plastic bottle (100 mls). Honey samples that were soft-set or crystalline were treated as described above before being analysed.

Preparation of English Chorley Honey (Code No. 261)

This honey was donated by Dr. Croft who upon request ensured that during its removal from the combs it was carefully manipulated with minimal heating. This was done so that the honey was subjected to conditions which would have little or no overall effect on the honey's consistituents and composition and this sample when received was stored at $4^{o}C$.

A portion equivalent to one-third of the total amount of honey received was subjected to the following conditions. These conditions mimicked those encountered after the normal extraction from the hive and thereafter processing by the beekeeper, followed by the commercial producer before bottling. This one-third portion of honey after removal from storage at $4^{o}C$ was equilibrated to room temperature over a period of two hours. Then the honey was strained through cheese cloth with gentle heating as described previously. Vigorous stirring of the strained honey was carried out with the aid of a spatula for about two to three minutes and then allowed to settle for a period of one hour. After this the honey was warmed at $60^{o}C$ for a period of twenty four hours.

The remaining two-thirds of the English Chorley honey that was not subjected to the conditions just described above was used as a control for the heat treated sample.

For the purpose of identification the heat treated one-third portion of English Chorley honey will be referred to by the Code No. 261a. Whereas the not heat treated two-third portion of English Chorley honey will be referred to by its original Code No. 261.

Subjective Analysis and Evaluation

The colour, appearances and odour of the U.K. survey and foreign and commercial samples were determined by sensory examination. The colours of the honeys ranged from a soft pale white crystalline appearance of the *Brassica* honeys to the dark brown almost black of the China buckwheat. Nearly all the samples received were either soft-set or crystalline with only a few that were liquid.

In general, the aroma of the samples ranged from a gentle pleasing fragrance of flowers and fruit blossom, especially the light colour honey, to the strong unpleasant of the dark colour honeys. The sign of fermentation was evident in some of the sugar fed samples and in some of the bulk honeys by the distinct smell of alcohol. An odour characteristic of hydroxymethylfurfural was evident in some commercial samples.

The honey samples were not tasted as the laboratory was within a building especially designed for radioactive analysis and any mouth pipetting or tasting was forbidden.

Furthermore, considerable experience was required in recognising the different aromas and flavours.

An example of the questionnaire sent to the participating beekeepers is given in Appendix II and their replies to questions No. 2, 3, 4, 5 and 6 have been given under their appropriate sections.

The Isolation and Concentration of Proteins in Honey

Ultrafiltration

The basic principle involved the separation of soluble low molecular weight components those less than 10,000 daltons from a solution containing both micromolecules and macromolecules. This separation is based on the use of a controlled pore membrane which permits passage of a defined range of molecular sizes. This separation is achieved by applying a positive pressure on the solution above a semi-permeable membrane.

The concentration of proteins present in honey was achieved by accelerated ultrafiltration using positive nitrogen pressure in an Amicon Standard cell, Model 402 of 400 ml capacity, fitted with a UM10 semi-permeable membrane filter whose molecular weight exclusion limit is 10,000 daltons.

Representative honeys from the foreign and commercial samples were examined for protein content; these samples were: Australia Banksia (Code No. 194), Canada (199), Chile (214), Hungary Polyflora (222), Mexico Yucatan (227), New Zealand (229), Yellow Box (246) and English pure Staffordshire (248).

Procedure

The procedure used by Bergner and Diemar (1975a) was followed with a few modifications. A sample of honey (25g) was weighed out and then diluted with 0.033M sodium phosphate buffer (50 mls) at pH 6.5.

This honey mixture was transferred to the Amicon standard cell. A pressure of four hundred and fourteen kilo Pascals (sixty pounds per square inch) was applied using a nitrogen cylinder. Portions (20 mls) of buffer were added when the solution in the Amicon standard cell reached 10 to 15 mls. This process was repeated until the filtrate gave a negative result on testing with Benedict's solution. This indicated that the reducing sugars and other soluble components including macromolecules of less than 10,000 daltons had been removed. The solution within the Amicon standard cell was concentrated to a volume of 10 mls. This protein concentrate from honey was then freeze-dried and the weight of the solid product was noted.

This dried residue was stored *in vacuo* in the presence of phosphorous pentoxide to prevent the process of hygroscopicity and microbial contamination.

It was found necessary to perform the filtration process at $4^{o}C$ in order to minimize the risk of bacterial contamination of the honey solution.

Quantitative Estimation of Proteins in Residues from Ultrafiltration of Honey

The Lowry Method

Proteins when reacted with Folin-Ciocalteau's reagent in the presence of alkaline copper solutions, that is, under conditions given by Lowry *et al.*, (1951) bring about a reduction of the phosphomolybdic-tungstic mixed acid by the loss of up to three oxygen atoms from the tungstate and/or molybdate ions. This mixed acid is the final chromagen and involves the following chemical species:

$3H_2O \cdot P_2O_5 \cdot 13WO_3 \cdot 5MoO_3 \cdot 10H_2O$

and

$3H_2O \cdot P_2O_5 \cdot 14WO_3 \cdot 4MoO_3 \cdot 10H_2O$

The result is the production of one or more of several possible reduced species which have a characteristic blue colour (λ_{max}, 745 - 750 nm and λ_{min} 405 nm). The copper ions present form chelates with the peptide bonds of the proteins thereby facilitating the transfer of electrons to the mixed acid chromagen, particularly in the vicinity of the amino acid functional groups. Thus increasing the sensitivity of the proteins. Hence, as the amino acids alter in concentration for each protein, so does the response. It is conventional to use a soluble protein-serum albumin as standard (Peterson, 1979).

Preparation of solutions and reagents

Solution A consisted of 2% (w/v) bisodium carbonate dissolved in 0.1M sodium hydroxide solution.

Solution B_1 consisted of 1% (w/v) copper sulphate ($CuSO_4 \cdot 5H_2O$).

Solution B_2 consisted of 2% (w/v) sodium potassium tartarate.

Reagent B was obtained by mixing equal volumes of solution B_1 and B_2.

Reagent C was obtained by mixing solution A with reagent B by the ratio 50:1 (v/v) and was freshly made when required.

Reagent D was obtained by mixing 1 volume of Folin-Ciocalteau's reagent with 2 volumes of water.

Procedure

The method of Lowry *et al.*, (1951) was used. Bovine serum albumin was used to prepare a series of protein standards covering the range from 20 to 260 µg of protein per ml. Portions of the residues from ultrafiltration of honey (0.1g) was weighed and dissolved in distilled water (5 mls) and each 1 ml of this solution was further diluted to 5 mls. Aliquotes (0.5 mls) of each of these diluted solutions, that is, the protein standards and honey concentrates were mixed with reagent C (5 mls) and left for ten minutes at 23°C, before adding reagent D (0.5 ml) and mixing immediately. After being allowed to stand at room temperature for thirty minutes, the absorbance of each solution relative to a blank, that is, all reagents but no protein, was read at a wavelength of 500 nm using a spectrophotometer. Furthermore, for protein standards at 20 µg per ml concentration and for honey samples with absorbance values of less than 0.04 at wavelength 500 nm, were also re-read at a wavelength of 750 nm. This was done to check for accuracy of concentration determined at the 500 nm measurements.

A standard calibration curve was obtained by plotting the absorbance measurements for each of the protein standards against their appropriate known concentration values. The concentration of proteins in the diluted honey residues after ultrafiltration were obtained from the standard curve.

The Determination of Proteins in Honey

Disc-Gel Electrophoresis

Proteins always contain amino acid residues with acidic and basic groups projecting from the polypeptide structure and these polar groups

ionise in aqueous solutions. At any given pH for the solution, the charge on the protein will depend on the number and pKa of the various side chains on the surface of the protein. The process of migration of the protein towards the electrodes, their direction and relative mobility when an electric field is applied is known as electrophoresis. The term relative mobility is a complex function including factors such as net charge, molecular size, and gel cross-linking. The effect of all these factors can only be determined by prior calibration.

Polyacrylamide gels are formed by the polymerisation of acrylamide monomer ($CH_2 = CH \cdot CONH_2$) in the presence of a co-monomer methylenebis-acrylamide (BIS) ($CH_2 = CO \cdot NH \cdot CH_2 \cdot NH \cdot CO \cdot CH = CH_2$) which acts as a cross-linking agent. Polymerisation only occurs after initiation by free radicals formed from ammonium persulphate, riboflavin or other compounds. N,N',N",N"'-tetramethylenediamine is added as an accelerator. Different pore sizes can be produced by varying the total acrylamide concentration, that is, the degree of polymerisation or chain length and also by varying the degree of cross-linking through changing the proportions of acrylamide and bisacrylamide. Electrophoresis carried out on these mixed polymers is referred to as polyacrylamide gel electrophoresis (PAGE).

Protein movement in individual gels is detected by staining with a suitable protein stain such as napthalene black or coomassie blue and then destaining by thorough washing of each gel with the appropriate media. Thus the relative movement of the proteins to the bromophenol blue dye can be measured before and after staining (Smith, 1976).

Preparation of buffers

Electrophoresis buffer consisted of Tris(hydroxymethyl)-methyl-amine (28.8g) dissolved in distilled water to a final volume of 100 mls at pH 8.5.

Gel buffer (0.38M tris-HCl, pH 8.9) consisted of tris (4.5g) dissolved in distilled water (50 mls) and adjusted to pH 8.9 and then made up to 100 mls.

Procedure

The procedure of Sargent and George (1975) was followed with a few modifications. Acrylamide (6.28g) and N,N'-methylene-bis-acrylamide (0.1428g) were dissolved in gel buffer (99 mls). N,N',N",N"'-tetramethylene diamine (30 µl) was added and the solution was mixed and filtered. Polymerisation was catalysed by the addition of a freshly prepared 7% (w/v) solution of ammonium persulphate (1 ml). This gel solution was carefully pipetted into the electrophoresis tubes to within 0.5 cm from the top. These tubes were maintained in a vertical position with their lower ends sealed with serum caps. Distilled water was then carefully layered above the gel so as to exclude air and also to obtain a flat surface. The process of gelling was, normally, completed in thirty minutes and gel formation was evident when a distinct division appeared between the upper water layer and the gel below. The water layer was removed and replaced by the electrophoresis buffer. These tubes were then placed in the electrophoresis tank taking care to exclude air bubbles from the bottom of each of the tubes. Electrical connections were so arranged that the upper surface of the

tube was the cathode. Each buffer compartment was filled with electrophoresis buffer (250 mls). The residue from ultrafiltration of honey (0.01g) was dissolved in distilled water (5 mls), and 100 µl of this solution was applied together with one drop of glycerol and a drop of 0.05% (w/v) bromophenol blue solution. Electrophoresis was performed under conditions of constant current at 50 volts initially to allow the sample to enter the gel. The voltage was then increased so as to maintain a current of 4 milliamps per tube for a period of one hour.

After electrophoresis the gels were removed from the tube by rimming. This involved forcing water round the circumference of the cast gel using a hypodermic needle and syringe. The cast gels were then transferred to a staining solution of napthalene black 12B (w/v) in 7% acetic acid for thirty minutes. The stained gel was carefully washed in distilled water and then destained in 10% acetic acid solution. The regions not containing the protein became clear. The destained gels were stored in 10% acetic acid solution. The distance moved by the protein bands, bromophenol blue and the gel length were measured for each gel.

Detection of Honey Enzyme Activity on Polyacrylamide Gels

The presence of enzymes such as diastase, glucose oxidase and invertase in honey samples was investigated by performing polyacrylamide gel electrophoresis as described above except that the stage involving the application of marker dye bromophenol blue and staining of the gels with napthalene black was not carried out. Instead picric acid was used as an indicator for the presence or absence of glucose. Glucose will bring about the reduction of the yellow picric acid to the

orange red picramic acid. During the catalytic action of the enzyme glucose oxidase, glucose is consumed where as glucose is a by-product resulting from the catalytic action of the enzymes diastase and invertase on their substrates starch and sucrose, respectively. Hence, after electrophoresis the replicate gels containing the honey proteins were incubated in either 1% solutions of starch, glucose or sucrose at 37°C for a period of one hour. Also samples of the enzymes mentioned above were obtained from microorganisms or other sources commonly used in biochemistry laboratories were also applied to the gels on their own and also mixed with honey sample. These gels were incubated in their appropriate carbohydrate solution as described above. After incubation the gels were removed from their carbohydrate solution and washed with distilled water to remove excess carbohydrate solution. These gels were then immersed in a dilute solution of picric acid and two drops of 4M sodium hydroxide solution was added. The tubes containing the gels and picric acid were warmed gently in a water bath at 60°C for twenty minutes. After this period of warming the tubes were removed from the water bath and the changes in colour on the gels in the form of bands were noted.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

By including detergents such as sodium dodecyl sulphate (SDS) during electrophoresis better results are obtained in terms of fractionation, resolution and most importantly the ability of the proteins that are now proportional to molecular weight. The polyacrylamide gel electrophoresis with SDS will be referred to as SDS-PAGE. At neutral pH, in a 1% SDS solution which was also 0.1M with respect to mercaptoethanol, most proteins bind to SDS and dissociate, disulphide linkages are broken

by the mercaptoethanol, secondary structure is lost and the complexes consisting of protein subunits and SDS assume a random-coil configuration.

Protein treated in this way behave as though they have a uniform shape and an identical charge to mass ratio. This is because the SDS bound per unit weight of protein is constant: 1.4g of SDS per g of protein. The charge on the protein is in fact determined by the SDS rather than the intrinsic charge of the amino acid residues. Thus the effective mobility is related only to molecular weight because of the molecular sieving property of the gel. By varying the amount of acrylamide and bisacrylamide the desired pore size can be obtained enabling the separation of molecules of molecular weight ranging from 10,000 to 100,000 daltons for 10% T cross-linking to 20,000 to 350,000 daltons for 5% T cross-linking (Sargent and George, 1975 and Smith, 1976).

Preparation of buffer and solutions

Gel buffer consisted of 0.2M sodium phosphate buffer at pH 7 and 0.2% sodium dodecyl sulphate.

Sample buffer consisted of 0.01M sodium phosphate buffer at pH 7 and 1% sodium dodecyl sulphate together with 1% β-mercaptoethanol.

Electrophoresis buffer consisted of gel buffer diluted 1:1 with distilled water.

Acrylamide solution (10%) consisted of acrylamide (22.2g) and N,N'-methylene-bis-acrylamide (0.6g) both of these were dissolved in distilled water (100 mls) and stored in a dark brown bottle at $4^{o}C$.

5% acrylamide solution was prepared by diluting the 10% acrylamide solution 1:1 with distilled water.

Procedure

The procedure of Weber and Osborn (1969) was followed with a few modifications. It was found that raw honey (2g) dissolved in sample buffer (4 mls) was suitable and this solution was incubated at 37°C for a period of two hours, to ensure complete cleavage by β-mercapto-ethanol of all the disulphide bonds present in the honey proteins. Moreover, it was found that residues from ultrafiltration of honey (0.05g) dissolved in sample buffer (1 ml) and then incubated at 37°C for two hours, were also suitable.

Gel buffer (15 mls) was mixed with acrylamide solution (13.5 mls) and deareated. 1% ammonium persulphate solution (1.5 mls) freshly prepared was added, followed by N,N',N'',N'''-tetramethylenediamine (25 µl) and mixed thoroughly. This gel solution was activated and cast as previously described.

Solutions of sample buffer and English Chorley honeys Code Nos. 261 and 261a were prepared as described above and also similar solutions of raw honey and residues from ultrafiltration of honey (100 µl) were each mixed with a drop of 0.05% bromophenol blue solution together with a drop of glycerol, were then applied to the gels.

Electrophoresis was performed at a constant current of 2 milliamps per tube for a period of forty minutes in order to allow the sample to enter the gel. Then the current was increased so as to maintain a constant current of 6 milliamps per tube for a period of four hours.

After electrophoresis and the removal of the gels, the distance moved by the marker was measured. The gels were stained in a solution of coomassie brilliant blue (1.25g) dissolved in a mixture of 50%

methanol (454 mls) and glacial acetic acid (46 mls), overnight. The gels were then washed with distilled water and destained in destaining solution. This being a mixture of glacial acetic acid (75 mls) and methanol (50 mls) and water (895 mls). Frequent changes of destaining solution were made until the regions not containing the proteins became clear. Then the length of each gel was measured and the distances moved by the protein bands were also measured from the top of the gels. The resolution of the stained protein bands were sharp and the bands occuring close together, that is, separated by a distance of 0.1 cm were clearly visible. For broad bands, 0.5 cm and wider, the position of greatest density was obtained by averaging the distances moved by the leading and trailing edges of the protein band. The accuracy of measurement of distances moved by the bands was on average ±0.1 cm.

Gel scanning

The destained gels were placed in gel scanning tubes. These tubes were then filled with distilled water. Before sealing the tubes with rubber stoppers it was ensured that there were no air bubbles present. The air bubbles were removed by gently tapping the tubes and topping up with distilled water. The tubes were then sealed with rubber stoppers. These tubes containing the gel were then placed horizontally on the scanner, usually with the top of the gels entering the scanner first. Prior to this the gel scanner was allowed to warm up and the necessary adjustments made as described by the manufacturers manual. The gels were then scanned at a wavelength of 560 nm and at a speed of 6.33 mm per minute. The absorbance values for each band as

displayed by the absorbance meter were noted on the chartpaper. Gels were usually scanned a week after the destaining process was completed.

The relative position of the protein bands obtained on the gels suggest that the molecular weights of nearly all the proteins present in the honeys examined ranged from 60,000 to 20,000. However, in some honeys there were bands which occurred very near the top of the gels thus suggesting the presence of high molecular weight proteins.

Determination of the Free Ninhydrin Positive Substances of Honey

Although the presence of free amino acids in honey have been reported by various researchers, the presence of amines, amides, imino acids, small peptides and other substances forming ninhydrin positive complexes have also been reported, Davies (1975) and Siddiqi (1981). Hence in the present study these compounds will be referred to as ninhydrin positive substances. However, of these, the free amino acids are considered to be of major importance.

Separation and Concentration of the Free Ninhydrin Positive Substances of Honey

Ion-exchange chromatography

The principle of ion-exchange chromatography is that charged molecules absorb onto an ion-exchanger reversibly so that molecules can be bound or eluted by changing the ionic or pH environment.

An ion-exchanger is usually a three dimensional network or matrix that contains covalently linked charged groups to which counter ions are electrostatically bound using conditions that give stable and tight

binding. Then using a different ionic and/or pH conditions the ions are eluted off the ion-exchanger according to their extent of charge. Cation exchangers contain Resin-SO_3H and counter ions such as H^+, Na^+ etc., are retained and eluted with alkaline buffer. Anion exchangers contain Resin-quantanary basis and similarly, counter ions such as OH^-, Cl^- etc., are retained and eluted with acid buffers.

Procedure

The procedure for the separation and concentration of amino acids from a honey sample as described by Siddiqi (1981) was adopted and several modifications and improvements were incorporated to ensure quantitative determinations. The modifications were made as a result of preliminary trials and recovery experiments. This final modified procedure was then applied to solutions containing known amounts of amino acid standards or honey plus amino acid standards in order to check for recovery. The composition and concentration of these solutions are given in Table 5. The assessment of recovery of amino acid from the ion-exchange resin was carried out by applying the modified method in whole or in part to the amino acid solutions A and B as given in Table 6. These solutions A, B and S1 contained known weighed amounts of thirteen amino acids dissolved in either 10% (v/v) propan-2-ol/0.5M hydrochloric acid or a carbohydrate solution containing 33% glucose and 33% fructose.

Once satisfactory recoveries were obtained the modified procedure was then applied individually to all the two hundred and fifty six samples of honey, in a like manner. Further, for a few randomly chosen honey amino acid concentrates were preliminarily checked for the presence

TABLE 5

Concentration of amino acids in solutions A, B and S1

Amino acids	Concentration of amino acids in solution as prepared by weighing (nMoles per ml) A*	B**	(nMoles per 0.6 mls) S1***	(nMoles per ml B1****
Lysine	394	394	1180	483
Aspartic acid	418	418	1250	513
Threonine	502	502	1510	616
Serine	380	380	1140	466
Glutamic acid	596	596	1790	730
Proline	4960	4960	14880	6080
Glycine	618	618	1850	757
Alanine	474	474	1420	580
Valine	324	324	970	397
Isoleucine	398	398	1190	488
Leucine	388	388	1160	476
Tyrosine	650	650	1950	797
Phenylalanine	814	814	2440	998

* - Solution A was made up in 10% propan-2-ol/0.5M hydrochloric acid solution.

** - Solution B was made up in 33% glucose plus 33% fructose solution.

*** - Solution S1 was made up in 10% propan-2-ol/0.5M hydrochloric acid solution.

**** - Solution B1 concentration values given are those from the total 10.6 ml, that is, Solution B (10 mls) + Solution S1 (0.6 mls).

TABLE 6

Separation and concentration of amino acids by ion-exchange chromatography on solutions of amino acids and honey samples

No.	Amino acid solutions or honey samples	Amount used (mls or g)	Addition of S1* (mls)	IXC[1]	AAAA[2]
1	Solution A*	10.0	-	-	+
2	Solution A1*	10.0	-	-**	+
3	Solution A2*	10.0	-	+	+
4	Solution B*	10.0	-	+	+
5	Solution B1*	10.0	0.6	+	+
6	U.K. Survey Sample Code No. 174	10.0	-	+	+
7	U.K. Survey Sample Code No. 174	10.0	0.6	+	+
8	China light amber Code No. 211	10.0	-	+	+
9	China light amber Code No. 211	10.0	0.6	+	+
10	Mexico Yucatan Code No. 227	10.0	-	+	+
11	Mexico Yucatan Code No. 227	10.0	0.6	+	+

* - For amino acid composition and concentration in solutions A, A1, A2, B, B1 and S1 refer to Table 5.

** - The technique of ion-exchange chromatography was not performed but the solution (10 mls) was evaporated to dryness as described in text using vacuum pump and liquid nitrogen.

1 - IXC = modified ion-exchange chromatography procedure.

2 - AAAA = Automatic Amino Acid Analysis procedure.

\+ = relevant experimental procedure performed.

\- = relevant experimental procedure not performed.

of proteins using the Bio/Rad micro assay. This involved the addition of 1 ml of coomassie Blue G-250 based reagent to 1 ml of honey amino acid concentrates. Then absorbances measurements at 595 nm were obtained. Similarly, absorbance measurements at 595 nm were also obtained for amino acid concentrates of amino acid solutions A and B, Table 5. A standard reference protein, bovine serum albumin, at 20 µg per ml concentration was also similarly assayed. All assays were carried out in duplicate. The results indicated that a de-proteinization step was not required.

Furthermore, at random periodical intervals during the processing of honey samples, recoveries from solutions of amino acids of similar composition as those given in Table 5 were checked to ensure similar consistency. Also, thirteen amino acids of solution S1 was added to honey samples to check for recoveries and the effects of other honey consistituents on the added amino acids.

Preparation of Resin for Binding

The viscous property of honey required the use of a large mesh size cation exchange resin, Dowex 50-X8 20 - 50 US mesh (Na). This resin facilitated the binding of the free amino acids and other similar compounds present in honey. This resin was initially washed in portions (500 mls) of distilled water until the eluents were no longer coloured by the resin. This suspension was then allowed to settle and stored in distilled water (500 mls). To this washed resin (10g) 6M hydrochloric acid was added and the mixture was stirred for ten minutes to protonate the resin. The protonated resin was washed with several portions of distilled water (100 mls) until the washings were no longer acidic.

Ion-exchange Chromatography Procedure

A prepared sample of honey as described before was used. Honey (10.0g) was weighed accurately and mixed thoroughly with distilled water (10 mls) with the aid of a glass rod. Thorough mixing was ensured by gentle warming to a temperature not exceeding 40°C. This honey solution was then added to the protonated resin until no more honey solution could be transferred by means of droplets. The beaker and the glass rod were then washed with distilled water (2 mls) and transferred by means of droplets until no more greasy traces of honey were left in the beaker or on the glass rod. This was repeated once more with a 2 ml portion of distilled water. Thus, quantitative transfer was ensured. The resin and honey mixture was stirred with the aid of a magnetic stirrer for fifteen minutes, for effective binding of the free amino acids to the resin. The supernatant containing the carbohydrates and other components of honey was removed carefully with the aid of a pasteur pipette to ensure no loss of resin. The amino acid bound resin was washed with distilled water (10 mls) with stirring for ten minutes and the supernatant removed by pipette. This process was repeated twice and all the supernatants were discarded.

A 7M ammonium hydroxide solution (5 mls) was added to the washed amino acid bound resin. This mixture was then stirred for ten minutes and then the supernatant was carefully pipetted off and retained. This process was repeated twice more. The resin was then washed twice with distilled water (2 mls) and all the supernatants collected were pooled.

The pooled supernatants were then evaporated to dryness using a vacuum oil pump at 1 mm of mercury pressure with the aid of a microsplash head fitted to a B14 cone (Washington, 1966). Liquid nitrogen was used

as a coolant in the vacuum pump traps to prevent the evaporated ammonia and water from contaminating the vacuum oil. The ammonia contamination of the extracted amino acids was reduced to a minimum. This was done by rinsing the microsplash head with distilled water (0.5 mls) twice to remove any splashings into the flask and evaporated in the usual manner. The final dried product was left overnight *in vacuo* at a pressure of 0.5 mm of mercury. Sodium hydroxide pellets were used as desiccant.

It was found that the dried product could not be effectively removed from the flask by physical manipulation. In order to ensure quantitative transfer of all the dried product the following procedure was utilized. Initially, 1 ml of 10% propan-2-ol/0.5M hydrochloric acid solution was added to the dried product flask and brought into contact with all the accessible inside glass wall of the flask. Dissolution was ensured with gentle warming and transferred to a clean pre-weighed 2.5 cm^3 drum vial with the aid of a pasteur pipette. All the solution in the flask that could be sucked up by the pasteur pipette was transferred and any solution that could be discharged from the pipette was discharged into the vial until no more visible traces were left either in the flask or on the inside glass walls of the pipette. This process was then repeated by adding 0.3 mls and finally by 0.2 mls of 10% propan-2-ol/0.5M hydrochloric acid to the flask and transferring. The 2.5 cm^3 drum vial containing the dried product - 10% propan-2-ol/ 0.5M hydrochloric acid (1.5 ml) mixture was weighed and the weight of the concentrated honey amino acid solution determined.

Each of these concentrated honey amino acid samples thus prepared were then stored in a refrigerator at 4^oC until required. After use

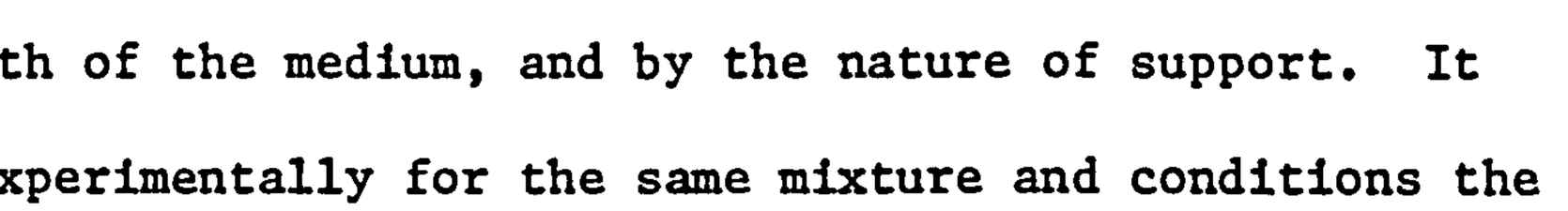
these samples were returned to the refrigerator until next required.

Examination and Identification of the Ninhydrin Positive Substances by a Combination of Paper Electrophoresis and Chromatography

Paper Electrophoresis

Biological molecules containing polar groups, -COOH or $-NH_2$, for example can loose or gain H^+ in solution to become the charged groups $-COO^-$ and $-NH_3^+$. These charged molecules can migrate in solution to the electrode of opposite polarity when an electric field is applied. Originally solutions were used but the process applies equally to an inert support containing a mobile and a bound water phase. The rate of migration of these molecules depends upon their size, shape and charge distribution and therefore the separation of two running compounds, referred to here as resolving power is influenced by the pH and ionic strength of the medium, and by the nature of support. It has been shown experimentally for the same mixture and conditions the resolving power decreases in the order PAGE > cellulose acetate > paper.

Amino acids are compounds which display both acid and base properties, that is, they are ampholytes and can ionise according to the pH of the solution. Thus the direction and extent of migration of ampholtyes are pH dependent and can be used to produce the required separation.



By employing electrophoresis at pH 1.9 all the natural amino acids except for the oxidation product cysteic acid exist as cations and therefore migrate towards the cathode. Cysteic acid has not been reported to be found in honey. The basis for the other part of the technique, paper chromatography, is given after the paper electrophoresis procedure.

Preparation of buffer

Electrophoresis buffer so-called consisted of a mixture of (98% - 100%) formic acid (104 mls) and glacial acetic acid (58 mls) and with distilled water was made up to two litres. The pH was checked and adjusted if needed to pH 1.9 ± 0.1.

Procedure

A sheet of Whatman No. 20 chromatography paper, 46 x 57 cm, was cut to give a sheet with the dimensions 40 x 57 cm and arranged with the longer edge on the right. The paper was then supported on glass rods to avoid contamination or contact with the bench whilst the honey concentrate or amino acid sample (25 µl) was applied by a micropipette, in stages with drying in between so as to maintain a constant minimum spot size of 0.5 cm diameter. The origin for the sample application was 10 cm from the 40 cm side and 14 cm from the 57 cm edge.

Electrophoresis buffer (250 mls) was placed in each of the buffer compartments. The loaded paper was then arranged in the electrophoresis tank so that the longer 57 cm edges dipped into the buffer compartments. The 57 cm edge nearest the origin of sample application was ensured to become the positive terminal when connected to the Vokam power pack. After moistening the paper evenly with electrophoresis buffer applied by a pasteur pipette all the electrical connections were made.

Electrophoresis was carried out at 250 volts for a period of four hours. The paper was carefully removed from the electrophoresis tank and then hung in a fume cupboard to dry at room temperature.

To this dried electrophoresis paper, thirty ninhydrin positive substances which according to the literature have been found to occur in honey were applied in solutions as reference standards. These solutions were spotted in two applications and dried so that the spot did not exceed 0.5 cm in diameter. The solutions, 25 µl of each of the ninhydrin positive substances, of different groups of standards arranged according to their RF values were applied at intervals of 1.5 cm on the 10 cm line between the sample origin and the 57 cm edge of the paper. These standard ninhydrin positive substance groups were:

(a) arginine, α-alanine, tyrosine, valine and phenylalanine;

(b) histidine, glycine, proline, methionine and leucine;

(c) lysine, serine, glutamic acid, tryptophan and isoleucine;

(d) Cysteine, aspartic acid and threonine;

(e) asparagine, hydroxyproline, γ-aminobutyric acid and pipecolic acid;

(f) ornithine and β-alanine;

(g) glucosamine, citruline, glutamine and α-aminobutyric acid;

(h) kynurine and β-aminobutyric acid.

Paper Chromatography

The technique of chromatography depends upon partition of a given compound between a stationary phase which is polar and a mobile covalent phase. At any instant, an equilibrium is set up and then displaced

until the tendency of the mobile phase to carry the components forward due to its solubility is balanced by the various attractive forces of the stationary phase and its solid support. On paper the major forces of attraction include hydrogen bonding and a weak ion-exchange mechanism involving hydroxyl and other groups on the cellulose.

The initial capillary attraction of the dry paper is responsible for the first 2 cm of solvent movement and thereafter in descending chromatography solvent movement is aided by gravity (Smith and Seakins, 1976 and Hais and Macek, 1963).

Preparation of solvent

The butanol-acetic acid-water solvent mixture (BAW) was prepared according to Siddiqi (1981). This solvent mixture consisted of butan-1-ol (200 mls) and glacial acetic acid (30 mls) and distilled water (75 mls) at pH 4.3.

Procedure

Descending chromatography was performed in a direction at right angles to the origianl electrophoresis using a Shandon Chromatank. The BAW solvent (150 mls) was placed in the solvent compartment and the bottom short end of the paper was dipped into the solvent. The chromatography paper was then hung in the chromatank and development with this solvent was allowed for twenty four hours. The paper was removed and then hung to dry in a fume cupboard, overnight.

Detection of the Ninhydrin Positive Substance

The formation of colour derivatives by the reaction between amino acid and ninhydrin is a universal method for detecting the presence of amino acids and similar related compounds. The reaction mechanism of

the colour formation has been disputed by various research workers proposing different pathways for the formation of the colour derivatives (Hais and Macek, 1963 and Metzler, 1977).

The use of metal chelates enables the enhancement of sensitivity and stability of the formation of colour complexes (Haworth and Heathcote, 1969).

Preparation of solutions

The ninhydrin reagent was prepared according to Haworth and Heathcote (1969).

Cadmium acetate stock solution was prepared by dissolving cadmium acetate (2g) in glacial acetic acid (40 mls) and distilled water (200 mls). Ninhydrin reagent was prepared by dissolving ninhydrin (2g) in cadmium acetate stock solution (24 mls) and propanone (200 mls). This mixed reagent was stored in a refrigerator until required and in any case not longer than seven days.

Procedure

Ninhydrin positive substances were detected on the chromatogram by dipping it in the cadmium acetate-ninhydrin reagent. The chromatogram was then hung in a fume cupboard to dry at room temperature. After some three hours the spots due to the high concentration of ninhydrin positive complexes became visible and these were outlined. The chromatogram was then stored in a dark sealed cylinder with a test tube of concentrated sulphuric acid (5 mls), to achieve an ammonia free environment. Each sheet was then examined every day for the next three days and then a week later to record the colour and position of the resulting ninhydrin positive complexes. This chromatogram was either

photographed or traced. Considerable experience was required to recognise the patterns of the ninhydrin positive complexes as these differed in kind and concentration.

Quantitative Analysis of the Free Amino Acids Using the Automatic Amino Acid Analyser

Amino Acid Analysis

The technique of ion-exchange chromatography followed by the identification and quantitative determination of individually eluted ninhydrin positive compounds can be automated. The quantitative determination of individual amino acid can be evaluated photoelectrically by measuring the absorbance of the ninhydrin-amino acid colour complex formed at a given wavelength. This being 570 nm for amino acids and 440 nm for imino acid, proline. The absorbance measurement can then be compared with that obtained with a corresponding amino acid standard of known concentration.

It is essential to prevent diffusion and mixing after separation on a capillary column filled with high resolution ion-exchange resin spheres of diameter 6×10^{-6} m. In general the separation or resolution is proportional to the column length and inversely proportional to the cross-sectional area and bead diameter. However, as the bead diameter decreases, the applied pressure needs to be increased substantially in order to maintain an adequate flow rate. The other major requirements are repetitive and constant sample application, consistent mixing of reagents and sample especially as the coloured products of the reaction may be measured before a true equilibrium is reached. These conditions are approximately satisfied in most commercial instruments.

Procedure

To the concentrated honey amino acid sample (25 µl) an internal standard solution of norleucine (25 nMoles) in 0.1M hydrochloric acid buffer was added. From this mixture duplicate samples (20 µl each) were applied to the analyser by first being loaded onto an equilibrated cartridge containing resin. The samples were then eluted on to a two column system containing ion-exchange resin. One column separates the basic amino acids while the other separates the acidic and neutral amino acids. Citrate buffer pH 5.25 for the basic amino acids and pH 3.25 and pH 4.25 for the acidic and neutral amino acids, were pumped at high pressure by means of two positive displacement pumps through these columns. These columns were operated at constant temperature of 60^{o}C by means of a water bath and a circulator.

The samples once eluted off the column were then mixed in a mixing coil with discrete quantitites of 1% ninhydrin-hydrazine reagent. This was then passed through a heating coil maintained at 95^{o}C for the colour reactions to take place. This solution was finally passed through the colorimeter which housed two flow cell assemblies with mounted interference filters of different wavelengths at 570 nm and at 440 nm. The output from the colorimeter was then recorded on a chart recorder in the form of peaks which are characteristics for each different amino acids. The normal run detecting seventeen standard amino acids at 25 nMoles concentration, each programmed cycle was completed in approximately two hours.

The concentration (nMoles) of each amino acid was then determined from the area under the trace response curve in comparison with the known standards obtained on chart paper. This was done by using the

formulae derived as shown in Appendix III. Hence, the concentration of amino acids per unit sample weight was calculated.

Quantitative Microscopical Analysis of Honey

Microscopical Analysis

The mature pollen grain is usually characteristic of its floral source, and differences between grains as to size, shape, surface contours and apertures can lead to identification. See, for example, Faegri and Iverson (1975), Moore and Webb (1978) and Sawyer (1981).

Light microscopy at a medium magnification of about 400X, is made easier by enhancement of the pollen grain features by selective staining with dyes, for example, basic fushin. Research has shown that this dye selectivity stains the polymerised series of hydroxy-carboxylic acids found in the exine sporopollenin of pollen (Stanley and Linskins, 1974). However, exines of different species vary in their response to stain. This suggests that sporopollenin is variable either in relative proportions of different moieties, or in the degree of cross-linking, or in the extent of the masking of reactive groups (Heslop-Harrison, 1971).

Moreover, identification of pollen source to the species level is not always possible even under ideal conditions. For pollen that has been collected by the bee stored in the hive and subject to great osmotic pressures generated by honey ready identification to *genus* level is possible (Howells, 1969). Also in some cases, especially pollen in honey of foreign origin, identification was possible to plant family level only (James, 1969).

Procedure

The method for the preparation of the microscope slides of pollen grains for the microscopical examination of honey, was according to those techniques described by Louveaux, Maurizo and Vorwohl (1970) and Sawyer (1981).

Preparation of the glycerine gelatin stains

Gelatin (7g) was dissolved in distilled water (42 mls) by warming on a hot water bath. To this gelatin solution glycerine (50 mls) and phenol (0.5g) was added with stirring.

Basic fuschin (0.1g) was dissolved in 70% ethanol (1 ml) and distilled water (160 mls).

Two grades of stained glycerine gelatin were prepared in the following way. To one glycerine gelatin (30 mls) basic fuschin solution (1.5 mls) was added and labelled as the 'Light coloured' stain. The other glycerine gelatin (30 mls) basic fuschin solution (3.0 mls) was added and labelled as the 'Dark coloured' stain.

Preparation of the microscope slides of the pollen grains

Honey (10g) was dissolved in distilled water (20 mls) by gentle warming. This honey solution was then centrifuged at 2500 revolutions per minute for ten minutes. The supernatant was decanted off carefully ensuring no loss of sediment. The sediment was dispersed in distilled water (10 mls) and re-centrifuged so as to remove traces of honey sugars. The supernatant was carefully pipetted off using a pasteur pipette. The sediment was transferred on to a microscope slide, as completely as possible, and spread over an area 15 x 15 mm by means of the tip of a pasteur pipette. The microscope slide with the pollen smear was then placed in an oven maintained at 40^{o}C, to dry.

Staining and mounting of the pollen smear

After drying the pollen smear was stained with two drops of the basic fuschin stain. Pollen smears containing little pollen were stained with the light coloured stain. For pollen smears containing a lot of pollen the dark coloured stain was used. Moreover, for the intermediate pollen smears a combination of both the light and dark coloured stains were used.

A coverslip was carefully positioned near the edge of the pollen smear and lowered carefully with the help of a pair of twizzers. In this way no air bubbles should become trapped between the coverslip and the slide. A uniform distribution of the stain between the coverslip and the slide was ensured by placing the slide in an oven maintained at 40^{o}C for ten minutes. Excess stain was removed by gently pressing the coverslip with a tissue paper and left overnight to set.

The coverslip was then sealed along the edges with a clear nail varnish to prevent the glycerine gelatin stain from becoming infected with bacteria or fungus.

Identification of Pollen Grains

Pollen grains were identified by using reference slides prepared by Ray Williamson containing thirty four different pollen grains, in conjunction with the identification key, index cards and microphotographs of pollen grains prepared by Sawyer (1981) and diagrams of pollen grains by Sawyer (1975). Also fresh pollen from known sources were obtained and standard reference sample slides were prepared.

The full list of all the pollen grains that were identified in the two hundred and fifty six samples of honey have been given in Appendix VIII.

In this list the identity of each pollen was specified to three classification levels, that is plant family followed by genus and then the species. Also given in this list for each pollen botanical name the equivalent common English name. In most cases the identity to a group of related plant species was possible, that is, genus; for example the *Brassica* related species pounded considerable problems of identifying species by pollen alone. Listed in Table 7 are the six European *Brassica* species out of the nine described by Crane, Walker and Day (1984) as important world honey sources. Pollen grains from closely related species cultivated nearby are likely to be present in a honey sample. For this reason and others discussed elsewhere, pollen identity were limited to genus only and were quoted as such throughout the present analysis. However, an exception was made for the nearly related *Trifolium* species whose distinctive pollen grains were readily identifiable. Pollens especially in the foreign honeys whose identity could not be determined even at plant family level were classed as unidentified.

Counting of Pollen Grains

Pollen grains were counted in the visual field as seen through the objective eyepieces of the microscope. Counting was carried out usually near the edge of the pollen smear with at least two counts minimum at or near the centre. Between 200 - 300 total pollen grains were counted and presented into frequency classes for each samples of honey. This was according to Louveaux, Maurizo and Vorwohl (1970). The pollen grains identified were then distributed according to their percentages into frequency classes. The following terms were used for expressing the

TABLE 7

List of common European species of the genus *Brassica*

Plant family	Genus	Species	
		Botanical name	English* name
Cruciferae	*Brassica*	*Campestris*	Field Cabbage
"	"	*juncea*	Chinese Mustard
"	"	*napus var. napobrassica*	Shaljam
"	"	*napus var*** *oleifera*	Oilseed Rape
"	"	*nigra***	Black Mustard
"	"	*rapa*	Winter Rape

* - There are other English names besides the common names, these names have been listed by Crane, Walker and Day, 1984.

** - Adequate honey yields are obtained from these plants.

frequency classes. These were: 'Predominant Pollen', which represented forty five percent and over pollen present, 'Secondary Pollen' represented pollen present between sixteen and forty five percent, 'Important Minor Pollen' represented pollen present between three percent and fifteen percent and 'Minor Pollen' represent less than three percent pollen present.

Computation

The experimental data gathered for each of the two hundred and fifty six samples warranted the use of a computer for statistical analysis and interpretation. The Salford Prime 750 computer has available the Statistical Package for Social Sciences (SPSS) version 9. This package comprises of statistical programs for data analysis and was written for social sciences (Nie *et al.*, 1975 and Hadlaihull and Nie, 1981).

The SPSS package on the Salford prime computer reads from a file and writes to a file. The data information and the SPSS commands contained in such files were typed in at the available visual display units (VDU) terminal (Noland, 1982 and Eckersley, 1982 and 1984). The procedure for access to the prime computer, files, editing and carrying out the statistical analysis using the SPSS package have been listed below:

1. By pressing the RETURN key a few times the 'SALFORD PRIME - LOGIN PLEASE' appears on the screen. The user name followed by the password are typed. When the OK, appears on the screen access to the prime system is then available.

2. By typing ED and obtaining INPUT on the screen has enabled a file to be opened. Then the information to be entered into this file can be typed in a pre-determined order. The format of the arrangement of the data in the data files have been described in detail in Appendix IV.

3. When all the relevant data have been entered into the file. After the last line, by pressing the RETURN key and then once more the EDIT appears on the screen. At this point the filename is typed, for example, DAT1-UK. Then OK, appears on the screen

4. A print-out of the contents of this file can be obtained by typing in 'SPOOL DAT1-UK, -AT, LEAR' the computer will transfer the contents of DAT1-UK file to the Lear-Siegler line printer for printing.

5. Any errors in the DAT1-UK file can then be located on the print-out. Ammendments to the DAT1-UK file in the computer can be made by typing ED DAT1-UK which displays the contents of the file on the screen. By using the editor commands TOP, BOTTOM, NEXT, LOCATE, PRINT the errors can be rectified. When all the corrections have been made the file can be closed as described previously except the filename need not be typed.

6. Another file, the SPSS control file was created by following the opening of a file procedure as described for DAT1-UK. The structure and format of a SPSS control file has been given in Appendix VI(b) and the filename was TEST1. This SPSS control file contains a series of control statements, each specifies a command which is recognised and obeyed by the SPSS.

7. The TEST1 file can also be checked for errors and corrections made by using the SPOOL and EDIT commands as described previously.

8. The statistical analysis detailed in the SPSS control file TEST1 were carried out by typing 'SPSS TEST1,-LISTINGS RESULT1'. The -LISTINGS RESULT1 is defined as the output file which contains the results of the statistical analysis and is printed at the Lear-Siegler line printer. When the terminal session is completed by typing the command LO after an OK, on the screen the computer will log out the user from the prime system.

Hence in this way two data files were compiled, one contained the one hundred and ninty two samples of the United Kingdom survey honeys arranged in the numerical order of the code number given in Appendix I. The other contained the sixty four samples of foreign and commercial honeys arranged in an alphabetical order according to their known country of origin as given in Appendix I.

Statistical Analysis

In order to obtain understandable biochemical information from the vast amount of numerical data collected for the two hundred and fifty six samples of honey, the use and application of statistics was considered appropriate with reference to Davies (1975 and 1976), Gilbert *et al.*, (1981) and Davies and Harris (1982). Since the subject of statistics was not my field of expertise, the experience and advice of Dr. Pemberton, Department of Mathematics, University of Salford was sort to process the analytical information gathered.

After familiarisation with the relevant aspects of both biochemistry and statistics the use and application of Discriminant analysis was recommended by Dr. Pemberton. A subprogramme 'DISCRIMINANT' was available on the SPSS package for calculation of Mahalanobis distance between populations such as pollen groups. After my initial attempt using raw data as collected with one modification (refer to Appendix VI(b)) which specified the SPSS to classify honey samples into nine predominant type pollen groups, was considered to require much needed improvements. Upon the recommendations of Dr. Pemberton, modifications and improvements which enhanced the predictive classification of the SPSS considerably were carried out. These modifications and improvements were: square root transformation of amino acid concentration measurements and removal of small sample number groups, that is, less than ten samples per group and defining of unifloral and multifloral subclasses were carried out as described in Appendix V(a).

Copies of the raw data file labelled 'DAT1-UK' and the SPSS control file labelled as 'TEST1' (Appendix VI) were obtained by using the command 'COPYF filename 1, filename 2. Relevant changes to each of the copy SPSS control files was made by replacement of appropriate commands which enabled random selection of samples in a particular group for processing and classification (Appendix VI(c)), recoding of sample groups (Appendix VI(d)) and selection and allocation of honey samples into appropriate groups (Appendix VI(d)(ii), (e), (f) and Appendix VII). The details of these modifications, improvements and changes of command carried out in SPSS control file have been detailed in Appendix V and briefly outlined below.

Discriminant Analysis on the United Kingdom Survey Honeys

As it is understood in the application of computers to statistical problems the following definitions are accepted. Discriminant analysis is the process of computation which enables calculation of group boundaries within a multi-dimensional space which will minimise error of allocating a honey sample of unknown origin to a group. One specialised section of this field is that of canonical variates analysis. Canonical variates analysis is the determination of Fisher's linear discriminant functions which are linear combinations of amino acid concentrations. A number of these linear combinations are known as canonical variates, these give varying degrees of group separation. Thus, each sample has a value on each canonical variate which can be plotted on co-ordinate axis. The maximisation of the minimum distance between groups is defined by the complex variable known as Mahalanobis distance. This is a stepwise statistical method developed by Mahalanobis and is described by the term 'MAHAL' which is recognised and obeyed by the SPSS (Appendix VI(a)).

The U.K. survey data file containing all the biochemical and analytical information for each of the one hundred and ninety two samples of honey was required. The SPSS control file described and detailed in Appendix VI(c) was used for carrying out the discriminant analysis. The SPSS was initiated as described earlier.

From the microscopical estimation and subsequent classification of the distribution of the pollen grains in a honey sample were used for allocating samples into nine predominant frequency class pollen groups. The SPSS then predicted classification of honey samples on the basis of their amino acid concentration measurements.

In order to improve the classification of samples the number of major pollen groups were reduced from nine to five and modifications performed on the original amino acid concentration values. In the five major pollen groups, those honey samples representing unifloral pollen were also evaluated.

Discriminant analysis was performed on the honey samples representing each of the three major sources of honey in the United Kingdom. These three major sources were: *Brassica*, *Trifolium repens* and *Calluna*.

Discriminant analysis was also performed on the honey samples harvested in the years 1981 and 1982; samples which were heated or otherwise and samples which were sugar-fed or otherwise. Moreover, these three analyses were also performed on honey samples representative of the five individual pollen groups. Similar analysis was also performed on the combined effects of heating and sugar feeding on a sample of honey.

The details of the procedure of the statistical analysis listed above have been described in Appendix V(a).

Discriminant Analysis on the Foreign and Commercial Honeys

Discriminant analysis was performed using the subprogram 'DISCRIMINANT' on the foreign and commercial honey samples. The foreign and commercial data file containing all the biochemical and analytical information for each of the sixty four samples of honey was required.

For the United Kingdom the major sources of imported honey are the following countries: Australia, Canada, Mexico and New Zealand and sometimes China. Discriminant analysis was performed on the samples of

honey originating from the first four countries together with English honey samples. The SPSS control file detailed and described in Appendix VII(a) and the control statements given in Appendix VII(b) were used to perform discriminant analysis on the foreign and commercial samples.

Discriminant analysis was also performed to distinguish between the following groups: nectar and honeydew honeys; honeys containing pollen originating from the same plant family; samples whose country of origin was not known but were allocated a country code from which they were most likely to have originated on the basis of their pollen content and amino acid patterns and samples that were or were not processed.

The details of the procedure of the statistical analysis listed above have been described in Appendix V(b).

Discriminant Analysis on the Combined Honeys of the United Kingdom Survey and the Foreign and Commercial

The United Kingdom survey honey samples and the foreign and commercial honey samples were pooled together in a sequential order.

The data file containing the foreign and commercial honey samples was appended onto the tail end of the U.K. survey data file by typing the command 'COPYF FOREIGN AND COMMERCIAL DATA FILE, UK SURVEY DATA FILE (APPEND)', in at a visual display unit terminal.

In this statistical analysis either of the SPSS control files detailed in Appendix V(a) and VII(b) could be used. The only modifications required were to change the number of cases to 256 and to replace the five 'IF' control statements. These control statements were 56 and 60 in Appendix VI(c) and 54 to 59 in Appendix VII(c).

Discriminant analysis was performed to distinguish between the United Kingdom and the foreign and commercial honeys; between the U.K. survey and six other country groups; and between the three pollen groups *Brassica*, *Trifolium repens* and *Castanea* common to the combined samples.

The details of the procedure of the statistical analysis listed above have been described in Appendix V(c).

Note that the commands used to initiate the SPSS which were typed in at a visual display unit terminal were correct up to the end of July 1983. However, some of these commands have changed in context and format due to the updating and revision of the computer systems carried out at regular intervals.

R E S U L T S

CHAPTER 3

R E S U L T S

CHAPTER 3

The Colour and Aroma of Honey

The colour and aroma were assessed for the honey samples that were examined.

In assessing the colours of the various honey samples numerical values were not obtained via a spectrophotometric method as this was considered not suitable. The colours of the honeys were therefore determined by visual inspection and in this investigation covered a broad spectrum. Arranged in the order of increasing colour one has the nearly water white honeys produced by the plant genera of *Brassica*, *Trifolium repens*, *Rubus* and *Robinia*. These were followed by the white to light golden colours of the *Echium* honey and then the light ambers of the *Impatiens* and *Prunus/pyrus* honeys. The light to medium ambers were distinctive of *'Viguiera'* and *Lotus* honeys. The *Castanea* and the *Vicia* and also the honeydew honey were associated with light to dark amber colours. The golden yellow to dark amber were distinctive colours of the *Helianthus* honeys. The heather honeys were characteristically dark reddish brown in colour. While those of the *Fagopyrum* honeys were rather dark brown almost black in colour. Furthermore, some of the Australia, China, Chile, Hungary, India, Mexico and Spain honeys, the colours ranged from the extra light ambers to the medium and then to the dark red brown ambers.

Characterisation of the aroma of honeys proved difficult to define as considerable experience was required in judging the various fragrances.

However, it was not the purpose of the present study to assess honeys by sight and smell, although identification of source can be

quickly established to the satisfaction of a honey show judge seeing hundreds of samples of a variety at a time. Moreover, numerical values could not be evaluated since not all the volatiles and the non-volatiles associated with honey aroma have as yet not been isolated and identified.

Nevertheless, it was found that the light colour class honeys had sweet pleasant smell compared to the strong and characteristic smell of the darker honeys. Moreover, some honeys had distinct fragrances such as those of apples, garden flowers and citrus fruits. Some of the Australia, China and other foreign honeys had a distinct fragrance associated with eucalyptol. The aroma of pine and fir trees similar to that of turpentine was evident in some of the European honeys suggesting the presence of terpenes. A distinct aroma associated with wet wood or saw dust was evident in some of the U.K. survey samples. One of the Indian samples had a characteristic smell of sulphur compounds such as sulphur dioxide. It is known that in some countries the practice of burning sulphur to destroy colonies and then collecting the honey is still performed. Some of the honeys did not have any characteristic aromas while others were marred by the distinct smell of fermentation or that of burnt wax. Yet in others and especially in some commercially produced samples the aroma of hydroxymethylfurfural was evident. One sample which was commercially processed and of a controversial nature, had an aroma which was characteristic of engine oil used for lubricating machinery.

These personal observations were the impressions on initial examination of samples as received and may not be true for the sample as collected from the hive or yet from the sample after long term storage.

ANALYSIS OF PROTEINS

Isolation, Concentration and Estimation of Proteins in Honey

The presence of proteins in the honey residues after concentration of a sample of honey (25g) by the technique of ultrafiltration was determined by the method of Lowry *et al.*, (1951). The concentration of proteins in the honey residues were determined from the calibration curve obtained for the standard reference protein, bovine serum albumin as shown in Figure 2. The amount of protein present in the honey samples prior to concentration were evaluated and are given in Table 8.

The use of polyacrylamide gels was disappointing as most of the honeys gave a similar unresolved band about 4 cm long. Also, in cases of China buckwheat honey Code No. 209 and other relatively high protein honeys the whole gel became stained not due to overload. This technique was not suitable for further investigations. Therefore, the only use of straight PAGE was for location of enzyme activity in unstained gels. The application of purified laboratory grade enzymes on polyacrylamide gels and then location with napthalene black stain resulted in sharp definite bands. These enzymes were diastase, glucose oxidase and invertase. Similar band pattern was also observed for a mixture containing honey and the standard laboratory grade enzymes. The position and distance moved by each of these bands were measured from the top of the gels and have been given in Table 9.

Moreover, the presence of enzyme activity due to diastase, glucose oxidase and invertase in honey applied to polyacrylamide gels was examined by using picric acid as an indiactor as described. However, the presence of an orange red band due to the formation of picramic acid was detected on gels to which standard laboratory grade enzymes diastase and invertase

FIGURE 2

Calibration graph for reference protein bovine serum albumin for absorbance measurements at wavelength 500 nm.

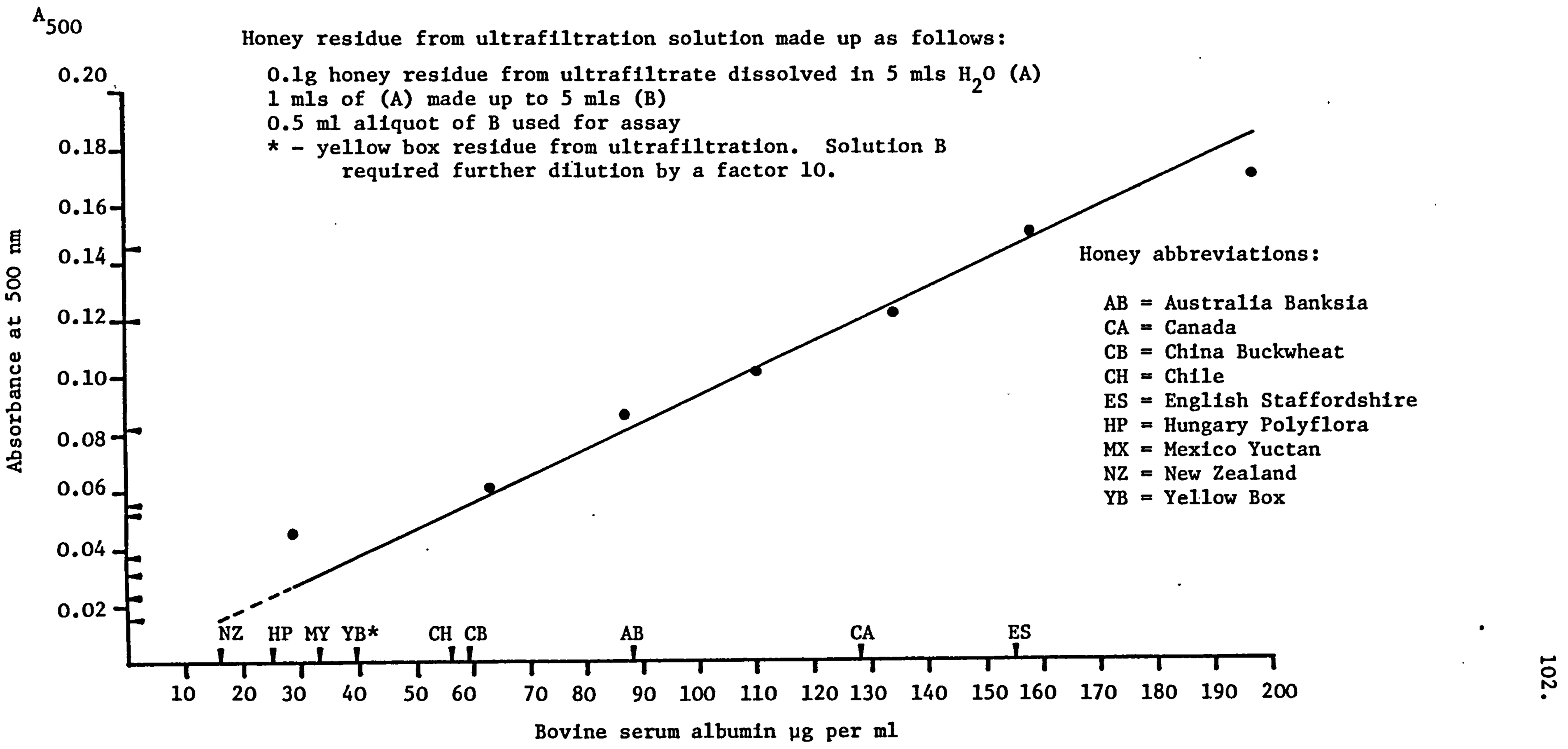


TABLE 8

Honey concentration by ultrafiltration

Honey source (Code No.)	Weight of concentrate (g) from 25 g of honey	Protein concentration* (mg) per 25 g of honey (and per 100 g of honey)
Australia Banksia (194)	1.322	58 (240)
Canada (199)	1.660	106 (424)
Chile (214)	0.462	13 (52)
China Buckwheat (209)	0.238	7 (28)
New Zealand (229)	0.253	2 (8)
Hungary Polyflora (222)	0.245	3 (12)
Yellow Box (246)	0.294	58 (240)
Mexico Yucatan (227)	0.244	4 (16)
English Staffordshire (249)	1.705	132 (528)

* = Determined by the Lowry et al., method - values represent milligram equivalent of bovine serum albumin used as reference protein

TABLE 9

Detection of presence of enzymes in honey by polyacrylamide gel electrophoresis

Enzymes*	Substrate (1%)(w/v)	Orange Red Band**	
		Presence	Distance from top of gel (cm)≠
Diastase[1]	Starch	Yes	4.4 to 4.9
Glucose oxidase[2]	Glucose	No	-
Invertase[1]	Sucrose	Yes	0.5 to 0.8

* - Standard laboratory grade enzymes obtained from microorganisms.

** - The reaction of picric acid with glucose under alkaline conditions resulted in the formation of picramic acid.

≠ - Average gel length for both picric acid and napthalene black stained gels, was 10.1 cm. Also, that the position of these bands were similar on gels which were stained with naphthalene black stain.

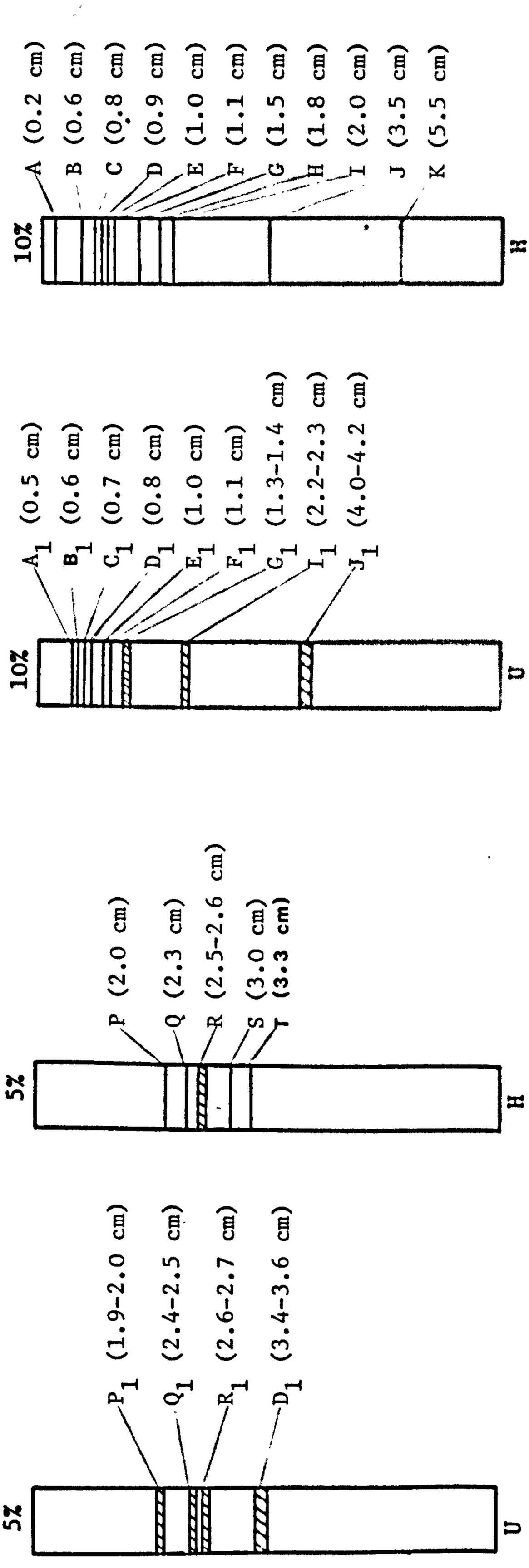
1 - The presence of enzymes diastase and invertase were not detected in honey but only in honey to which laboratory enzymes had been added. This mixture then analysed by electrophoresis.

2 - No orange red bands were detected for standard laboratory enzyme glucose oxidase suggesting the absence of glucose oxidase activity. Whereas preliminary tests for the detection of presence of the enzyme resulted in three bands (3.6-4.5, 5.0-5.5 and 6.8-8.0 cm) after staining with napthalene black stain.

had been applied but not however on gels to which glucose oxidase was applied. Although the orange red band was not evident on gels to which honey was applied, the orange red band was however detected on gels to which a mixture containing both the honey and standard laboratory enzymes were applied. The position and distance moved by these orange red bands were similar to those bands obtained as gels stained with napthalene black stain. These have been given in Table 9.

The presence of proteins in raw honey (2g) was detected by the technique of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. After electrophoresis and staining the protein bands which appeared on gels to which honey samples that were either heat treated or otherwise have been shown in Figure 3 and Tables 10 and 11. The position and distance moved by protein bands which appeared after staining on 5% and 10% polyacrylamide gels were measured from the top of the gel, this being the cathode or the negative end as shown in Figure 3. The protein bands were labelled alphabetically from the top. These bands corresponded to proteins whose molecular weights were largely between 60,000 daltons and 20,000 daltons, compared against reference standard proteins. An unusual pattern of the protein bands from that given in Figure 3 was observed on the SDS-polyacrylamide gels of national honey show sample Code No. 252, China buckwheat Code No. 209 and soyolk (soyabean protein). The unusual pattern has been shown in Figure 4.

The polyacrylamide gels after staining were also scanned on a gel scanner at wavelength 560 nm as described. A typical scan recorded onto a chart paper for both the 5% and 10% polyacrylamide gels have been shown in Figures 5 and 6 respectively. The relative position of the protein bands have also been labelled alphabetically according to those already shown in Figure 3.



261

of English Chorley honey code No.

on 5% and 10% polyacrylamide gels

FIGURE 3

Protein band pattern on

U = Unheated honey sample - English honey Chorley code No. 261

H = Heat treated honey sample English honey Chorley code No. 261 - band pattern was similar in all the honey samples that were examined.

NB Distance measurements of gel diagram.

TABLE 10

Protein bands which appear on a 5% polyacrylamide gel with a honey sample

Honey source (Code No.)	Protein bands				
	P	Q	R	S	T
Australia Banksia (194)	+	-	-	+	+
Canada (199)	-	+	+	+	-
Chile (214)	-	+	-	+	-
Mexico Yucatan (227)	-	-	+	-	+
Hungary Polyflora (222)	-	+	-	+	-
New Zealand (229)	-	+	+	+	-
Yellow Box (246)	-	+	-	+	-
China acacia (208)	-	+	+	+	-
China light amber (211)	-	-	+	-	+
English Staffordshire (249)	-	-	+	-	+
English Chorley (261)*	+	+	+	-	+

Protein bands P to T, refer to Figure 3.0
+ = Protein bands which were present on a gel
- = Protein bands which were not present on a gel

*English Chorley honey was subjected to heat treatment as described.

TABLE 11

Protein bands which appear on a 10% polyacrylamide gel with a honey sample

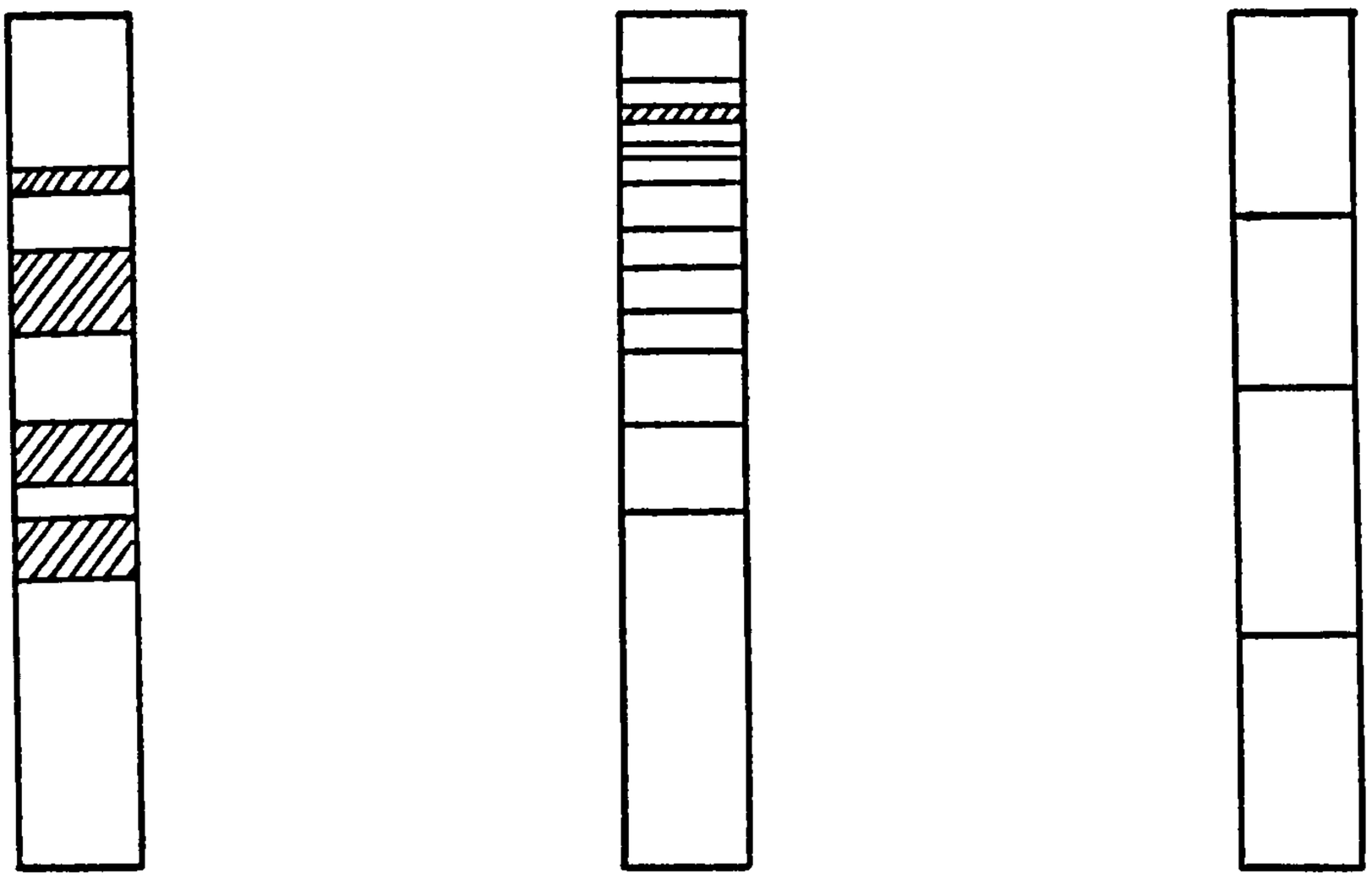
Honey source (Code No.)	Protein bands										
	A	B	C	D	E	F	G	H	I	J	K
Canada (199)	+	+	+	+	+	+	+	+	-	+	+
China acacia (208)	+	+	+	+	+	+	+	+	-	+	+
English Staffordshire (249)	+	+	-	+	+	+	+	+	-	+	+
China light amber (211)	-	-	+	+	+	+	+	+	-	+	+
Pure honey (PHFJH) (260)	-	-	+	+	+	+	+	+	+	+	+
Northern Ireland (259)	+	+	+	+	+	+	+	+	+	+	+
Lincolnshire (258)	-	-	+	+	+	+	+	+	+	+	+
Lancashire (Tarleton) (257)	+	-	+	+	+	+	+	+	+	+	+
Australia Banksia (194)	+	+	+	-	+	-	+	+	-	+	+
Chile (214)	+	+	-	-	-	+	+	+	-	+	+
Mexico Yucatan (227)	-	-	-	+	-	+	+	+	-	+	+
Hungary Polyflora (222)	-	-	-	+	-	+	+	+	-	+	+
New Zealand (229)	-	-	-	+	+	+	+	-	-	+	+
Yellow Box (246)	-	-	-	+	+	+	+	-	-	+	+
English Chorley (261)*	+	+	+	+	+	+	+	+	+	+	-

Protein bands A to K, refer to Figure 3.0
+ = Protein bands which were present on a gel
- = Protein bands which were not present on a gel

*English Chorley honey was subjected to heat treatment as described.

FIGURE 4

An unusual protein band pattern on 10% polyacrylamide gel.



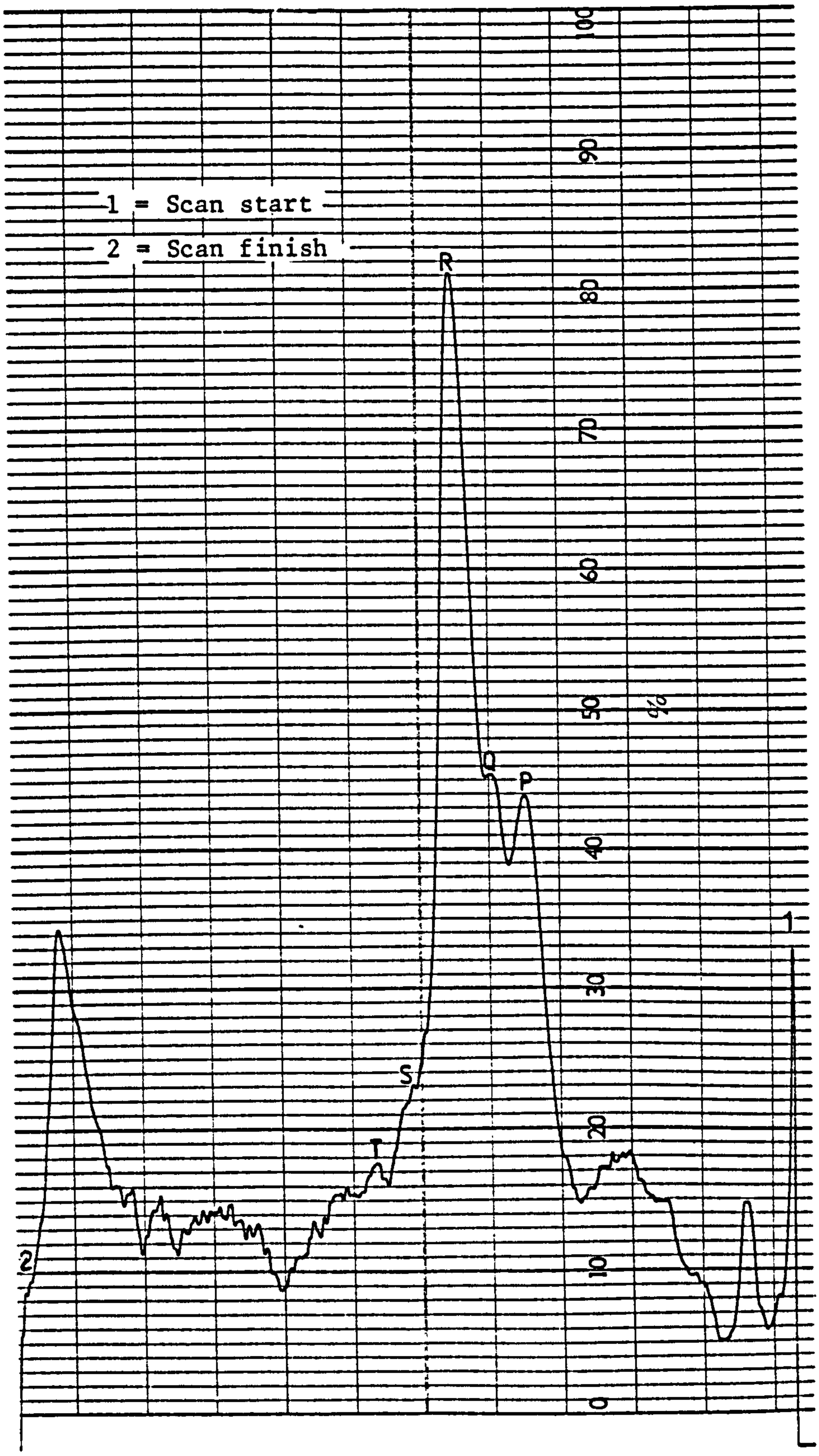
A = National honey show honey

B = Soyolk (Soya bean protein)

C = China buckwheat honey

FIGURE 5

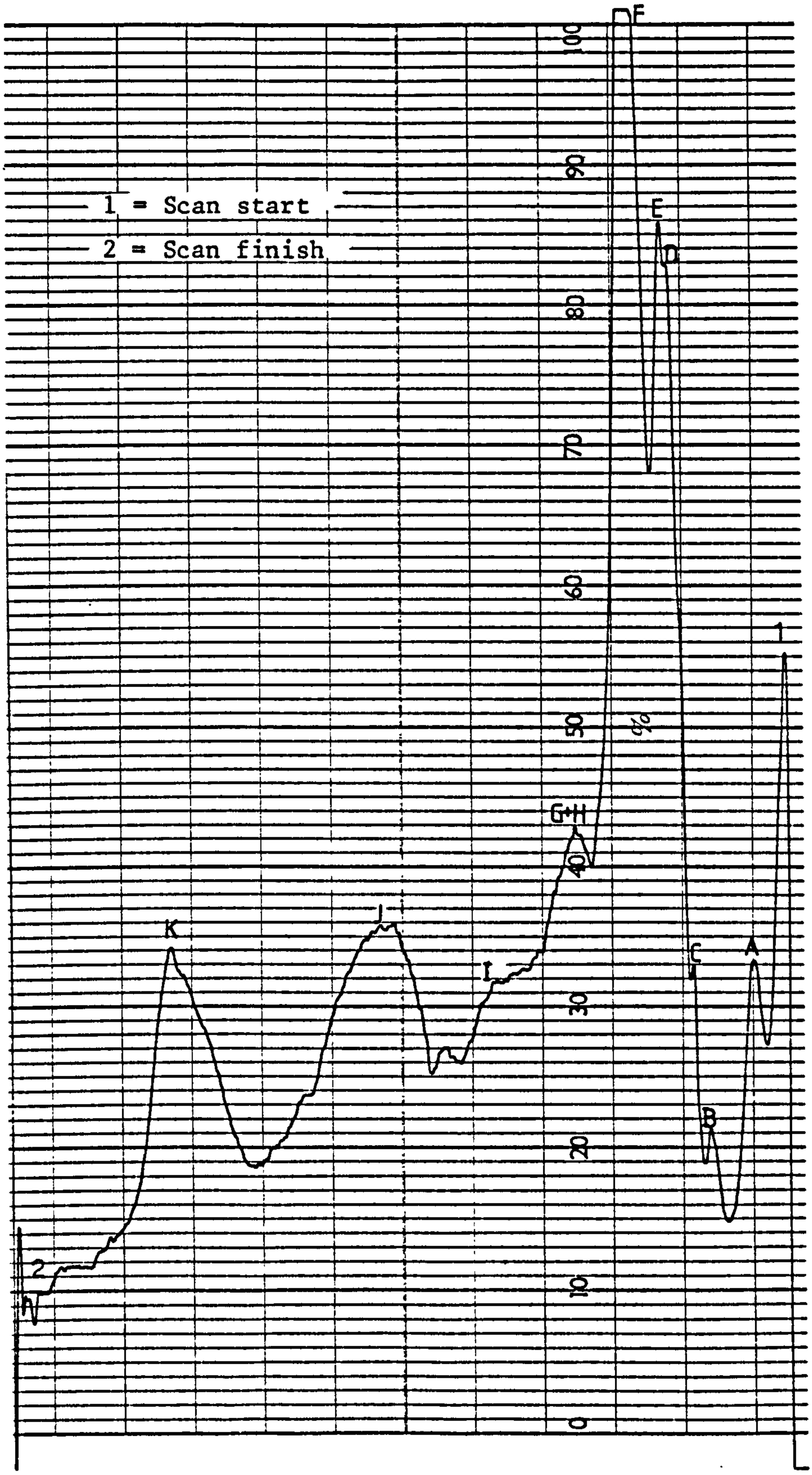
Gel scan of Canada sample No. 199 on 5% polyacrylamide gel.



P, Q, R, S, T - refer to Figure 3.

FIGURE 6

Gel scan of Canada sample No. 199 on 10% polyacrylamide gel.



A to K - refer to Figure 3.

Analysis of Ninhydrin Positive Substances and Pollens Present in Honey of Known Origin

The following analyses were carried out on each of the two hundred and fifty six samples of honey that were collected. These analyses were: the isolation and concentration of the free amino acids by the technique of ion-exchange chromatography; the separation and identification of the ninhydrin positive substances present in the amino acid concentrates by the method of paper electrophoresis and chromatography; the concentration measurements of the thirteen amino acids determined as the area under the appropriate peak of the chart recordings from the amino acid analyser; the microscopical determination of the pollen content of the individual honey samples and finally the statistical analysis of amino acid concentration measurements evaluated by a computer programme.

Analysis of Ninhydrin Positive Substances

The abbreviations utilised for the amino or imino acids or the ninhydrin positive substances that are detected in these honey samples or used as reference standards have been given in Table 12. These have been listed in an alphabetical order and coded numerically. These numerical codes were used for labelling the position of the ninhydrin complexes, as and when they occurred, after subsequent separation by the technique of paper electrophoresis and chromatography. A typical chromatogram showing the relative position of all these ninhydrin complexes have been illustrated in Figure 7. The ninhydrin complexes have been labelled both by numbers in the spot and by conventional abbreviated name of those identified on a line outside the spot. The low concentration ninhydrin complexes which were unidentifiable and which may be peptides were allocated a symbol and were labelled inside the spot.

TABLE 12

List of ninhydrin positive substances detected and their abbreviations

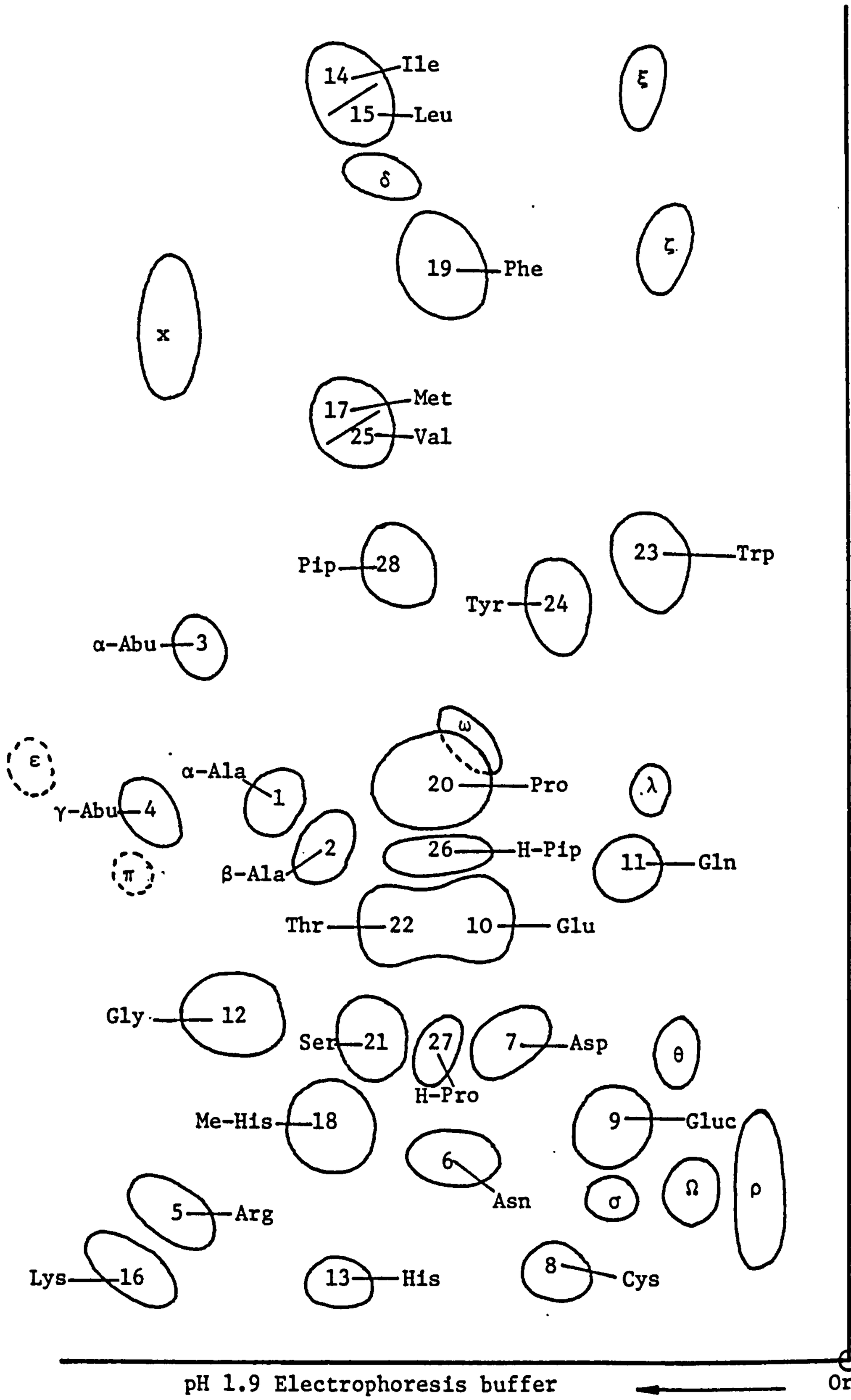
Code No.	Ninhydrin positive substances	Abbreviations
1	α-alanine *	α-Ala
2	β-alanine	β-Ala
3	α-aminobutyric acid	α-Abu
4	γ-aminobutyric acid	γ-Abu
5	Arginine	Arg
6	Aspargine	Asn
7	Aspartic acid	Asp
8	Cysteine	Cys
9	Glucosamine	Gluc
10	Glutamic acid	Glu
11	Glutamine	Gln
12	Glycine	Gly
13	Histidine	His
14	Isoleucine	Ile
15	Leucine	Leu
16	Lysine	Lys
17	Methionine	Met
18	Methyl-Histidine	Me-His
19	Phenylalanine	Phe
20	Proline	Pro
21	Serine	Ser
22	Threonine	Thr
23	Tryptophan	Trp
24	Tyrosine	Tyr
25	Valine	Val
26	Hydroxypipecolic acid	H-Pip
27	Hydroxyproline	Hyp
28	Pipecolic acid	Pip
29	ρ **	ρ
30	X	X
31	ξ	ξ
32	δ	δ
33	ζ	ζ
34	ε	ε
35	π	π
36	ω	ω
37	λ	λ
38	θ	θ
39	σ	σ
40	Ω	Ω

*1-28 identity confirmed

**29-40 identity not proven - could include peptides

FIGURE 7

Amino acids map of standard reference ninhydrin positive substances.



A series of experiments were carried out to check for the precision of the amino acid extraction method used and the recovery of amino acids from solutions containing known amounts of amino acids, refer to Table 5, and from randomly chosen honey samples. The amino acid concentration measurements were determined from the area under the response curve obtained on chart paper from the amino acid analyser. The initial area for a specific peak response curve for a given amino acid was estimated as being an equivalent to that of a triangle. The area of such a triangle was calculated as peak height multiplied by the width at half-height and for example of its use and for calculations, see Appendix III. Each chart was correlated with two or more calibrated solutions. It was usual to carry out a calibrating run then five samples and then a further calibration. All these were correlated to a constant internal standard level (norleucine). Discussion of the merits of this procedure and possible use of alternative internal standards are dealt with in the discussion. For reasons of brevity only the final results in terms of nanomoles per g of honey are presented for all the amino acid analyser determinations.

The amino acid concentration and percentage recovery values for each of the amino acid solutions and honey samples listed in Table 6 have been given in Tables 13 to 22. Also the amino acid concentrations and percentage recovery values for known amounts of amino acids added to the three honey samples are given in Tables 23 to 29. For reasons of clarity of presentation it was considered necessary that in the replicate analysis of all the honey samples examined, the average concentration values given were rounded off to the nearest whole number for values less than 500 nmoles per g of honey and to the nearest ten unit for

TABLE 13

Quintiplate analysis of the thirteen amino acids prior to experimentation on solution A

Amino Acids	Concentration of amino acids (nMoles per ml)						
	W*	A1**	A2**	A3**	A4**	A5**	Average***
Lysine	394	415	402	415	414	416	412
Aspartic acid	418	430	423	427	410	430	424
Threonine	502	506	496	500	503	495	500
Serine	380	385	382	387	387	397	388
Glutamic acid	596	607	604	600	594	592	600
Proline	4,960	5,022	5,010	5,088	5,047	4,990	5,030
Glycine	618	633	645	623	620	645	633
Alanine	474	477	473	480	466	464	472
Valine	324	320	317	316	327	316	320
Isoleucine	398	408	417	420	413	410	414
Leucine	388	403	407	400	403	400	403
Tyrosine	650	647	660	638	640	640	645
Phenylalanine	814	816	817	820	800	796	810

* = W – amount of standard amino acids in solution A determined by weight, refer to Table 5.

** = A1 to A5 – are the values for thirteen amino acids from five replicate analyses determined by the autoanalyser and technician for solution A using reference standards of 25 nanomoles of each amino acid. These conditions are used throughout and for experimental details refer to Table 6.

*** = Average concentration of the five A1, A2, A3, A4 and A5.

TABLE 14

Recoveries of amino acids in solution A from automatic amino acid analyser

Amino acids	Percentage recovery of amino acids (%)					
	A1	A2	A3	A4	A5	Average*
Lysine	105.3	102.0	105.3	105.0	105.6	104.6
Aspartic acid	102.8	101.2	102.2	98.0	102.8	101.4
Threonine	100.8	98.8	99.6	100.2	98.6	99.6
Serine	101.3	100.5	101.8	101.8	104.5	102.0
Glutamic acid	101.8	101.3	100.7	99.7	99.5	100.6
Proline	101.3	101.0	102.6	101.8	100.6	101.5
Glycine	102.4	104.4	100.8	100.3	104.4	102.4
Alanine	100.6	99.8	101.3	98.3	98.0	99.6
Valine	98.8	97.8	97.5	101.0	97.5	98.5
Isoleucine	102.5	104.8	105.5	103.8	103.0	104.0
Leucine	104.0	105.0	103.0	104.0	103.0	103.8
Tyrosine	94.5	101.5	98.2	98.5	98.5	99.2
Phenylalanine	100.2	100.4	100.7	98.3	97.8	99.5

* = Average percentage of the five A1, A2, A3, A4 and A5, also refer to Table 13 for corresponding concentration values determined from the autoanalyser.

TABLE 15

Quintiplate analysis of the thirteen acids recovered after evaporating solution A1

Amino Acids	Concentration of amino acids (nMoles per ml)						
	W*	E1**	E2**	E3**	E4**	E5**	Average***
Lysine	394	385	386	383	388	387	386
Aspartic acid	418	408	412	408	410	414	410
Threonine	502	493	496	492	498	494	495
Serine	380	372	375	376	374	377	375
Glutamic acid	596	590	592	588	593	590	590
Proline	4,960	4,860	4,870	4,830	4,850	4,880	4,860
Glycine	618	606	605	608	612	610	608
Alanine	474	464	465	467	470	464	467
Valine	324	320	318	317	320	322	320
Isoleucine	398	394	395	396	392	394	394
Leucine	388	388	385	385	386	387	386
Tyrosine	650	635	640	643	645	642	640
Phenylalanine	814	808	806	803	810	805	806

* = W - amount of standard amino acids in solution A1 determined by weight, refer to Table 5.

** = The series E1 to E5 are the values in replicate for the thirteen amino acids of solution A1 after evaporation and subsequent determination by the autoanalyser under standard conditions (see Table 13).

*** = Average concentration of the five E1, E2, E3, E4 and E5.

TABLE 16

Recoveries of amino acid in solution A1 from automatic amino acid analyser

Amino Acids	Percentage recovery of amino acids (%)					
	E1	E2	E3	E4	E5	Average*
Lysine	97.7	98.0	97.2	98.5	98.2	98.0
Aspartic acid	97.6	98.6	97.6	98.0	99.0	98.2
Threonine	98.2	98.8	98.0	99.2	98.5	98.5
Serine	98.0	98.6	99.0	98.4	99.2	98.6
Glutamic acid	99.0	99.3	98.7	99.5	99.0	99.0
Proline	98.0	98.2	97.4	97.8	98.4	98.0
Glycine	98.0	98.0	98.4	99.0	98.7	98.4
Alanine	98.0	98.0	98.5	99.2	98.7	98.5
Valine	98.8	98.0	97.8	98.8	99.4	98.6
Isoleucine	99.0	99.2	99.5	98.5	99.0	99.0
Leucine	100.0	99.2	99.0	99.5	99.7	99.5
Tyrosine	97.7	98.5	99.0	99.2	98.8	98.6
Phenylalanine	99.3	99.0	98.6	99.5	99.0	99.0

* = Average percentage of the five E1, E2, E3, E4 and E5, also refer to Table 15 for corresponding concentration values determined from the autoanalyser.

TABLE 17

Quintiplate analysis of the thirteen amino acids after ion-exchange chromatography of solution A2

Amino acids	Concentration of amino acids (nMoles per ml)						
	W*	I1**	I2**	I3**	I4**	I5**	Average***
Lysine	394	343	340	346	345	340	343
Aspartic acid	418	425	417	423	426	420	422
Threonine	502	492	488	485	498	487	490
Serine	380	378	372	380	374	370	375
Glutamic acid	596	590	588	596	590	588	590
Proline	4,960	4,830	4,820	4,840	4,820	4,830	4,830
Glycine	618	600	605	602	613	608	606
Alanine	474	467	468	475	480	473	473
Valine	324	316	315	318	320	322	318
Isoleucine	398	415	412	414	411	410	412
Leucine	388	394	395	395	405	392	396
Tyrosine	650	633	637	640	647	636	638
Phenylananine	814	810	815	805	807	804	808

* = W – amount of standard amino acids in solution A2 determined by weight, refer to Table 5.

** = The series I1 to I5 are the values in replicate for the thirteen amino acids of solution A2 after ion-exchange chromatography and subsequent determination by the autoanalyser under standard conditions (see Table 13).

*** = Average concentration of the five I1, I2, I3, I4 and I5.

TABLE 18

Recoveries of amino acid in solution A2 from automatic amino acid analyser

Amino acids	Percentage recoveries of amino acids (%)					
	I1	I2	I3	I4	I5	Average*
Lysine	87.0	86.3	87.8	87.6	86.3	87.0
Aspartic acid	101.7	99.8	101.2	102.0	100.5	101.0
Threonine	98.0	97.2	96.6	99.2	97.0	97.6
Serine	99.5	98.0	100.0	98.4	97.4	98.7
Glutamic acid	99.0	98.7	100.0	99.0	98.7	99.0
Proline	97.4	97.2	97.6	97.2	97.4	97.4
Glycine	97.0	98.0	97.4	99.2	98.4	98.0
Alanine	98.5	98.7	100.2	101.3	99.6	99.6
Valine	97.5	97.2	98.0	98.8	99.4	98.2
Isoleucine	104.3	103.5	104.0	103.3	103.0	103.5
Leucine	101.5	101.8	101.8	104.4	101.0	102.1
Tyrosine	97.4	98.0	98.5	99.5	97.8	98.2
Phenylananine	99.5	100.0	99.0	99.0	98.8	99.3

* = Average percentage of the five I1, I2, I3, I4 and I5, also refer to Table 17 for corresponding concentration values determined from the autoanalyser.

TABLE 19

Quintiplate analysis of the thirteen amino acids after ion-exchange chromatography of carbohydrate solution B

Amino acids	Concentration of amino acids (nMoles per ml)						
	W*	B1**	B2**	B3**	B4**	B5**	Average***
Lysine	394	350	360	344	362	353	354
Aspartic acid	418	433	433	430	426	430	430
Threonine	502	490	500	493	497	490	494
Serine	380	380	383	387	376	390	383
Glutamic acid	596	582	583	580	592	580	583
Proline	4,960	4,900	4,870	4,910	4,930	4,880	4,900
Glycine	618	620	620	615	607	612	615
Alanine	474	472	466	464	460	468	466
Valine	324	323	320	320	318	315	320
Isoleucine	398	402	395	405	412	400	403
Leucine	388	394	390	390	397	392	393
Tyrosine	650	640	630	640	650	660	644
Phenylananine	814	805	812	814	810	803	810

* = W - amount of standard amino acids in solution B determined by weight, refer to Table 5.

** = The series B1 to B5 are the values in replicate for the thirteen amino acids of solution B after ion-exchange chromatography and subsequent determination by the autoanalyser under standard conditions (see Table 13).

*** = Average concentration values of the five B1, B2, B3, B4 and B5.

TABLE 20

Recoveries of amino acids in carbohydrate solution B from automatic amino acid analyser

Amino acids	Percentage recoveries of amino acids (%)					
	B1	B2	B3	B4	B5	Average*
Lysine	88.8	91.4	87.3	92.0	89.6	89.8
Aspartic acid	103.6	103.6	103.0	102.0	103.0	103.0
Threonine	97.6	99.6	98.2	99.0	97.6	98.4
Serine	100.0	100.8	101.8	99.0	102.6	100.8
Glutamic acid	97.7	97.8	97.3	99.3	97.3	97.8
Proline	98.8	98.2	99.0	99.4	98.4	98.8
Glycine	100.3	100.3	99.5	98.2	99.0	99.5
Alanine	99.6	98.3	98.0	97.0	98.7	98.3
Valine	99.7	98.8	98.8	98.0	97.2	98.5
Isoleucine	101.0	99.2	101.8	103.5	100.5	101.2
Leucine	101.5	100.5	100.5	102.3	101.0	101.2
Tyrosine	98.5	97.0	98.5	100.0	101.5	99.0
Phenylalanine	99.0	99.8	100.0	99.5	98.6	99.4

* - Average percentage of the five B1, B2, B3, B4 and B5, also refer to Table 19 for corresponding concentration values determined from the autoanalyser.

TABLE 21

Quintiplate analysis of the thirteen amino acids after ion-exchange chromatography of carbohydrate solution B1

Amino acids	Concentration of amino acids (nMoles per ml)						
	W*	BS1**	BS2**	BS3**	BS4**	BS5**	Average***
Lysine	483	450	430	428	440	433	436
Aspartic acid	513	527	532	530	528	535	530
Threonine	616	608	600	597	605	604	603
Serine	466	463	460	458	468	460	462
Glutamic acid	730	722	724	720	716	720	720
Proline	6,080	5,920	5,970	6,020	6,040	5,990	5,990
Glycine	757	740	744	742	750	748	745
Alanine	580	567	576	580	570	573	573
Valine	397	388	395	392	390	387	390
Isoleucine	488	493	500	490	496	495	495
Leucine	476	482	486	478	480	484	482
Tyrosine	797	790	787	786	780	790	787
Phenylalanine	998	993	990	996	983	986	990

* = W - amount of standard amino acids in the mixture of solution B1 and S1 (0.6 ml) determined by weight, refer to Table 5.

** = The series BS1 to BS5 are the values in replicate for the thirteen amino acids of the mixture of solution B1 plus solution S1 (0.6 ml) after ion-exchange chromatography and subsequent determination by the autoanalyser under standard conditions (see Table 13).

*** = Average concentration of the five BS1, BS2, BS3, BS4 and BS5.

TABLE 22

Recoveries of amino acids in carbohydrate solution B1 from automatic amino acid analyser

Amino acids	Percentage recoveries of amino acids (%)					
	BS1	BS2	BS3	BS4	BS4	Average*
Lysine	93.2	89.0	88.6	91.0	89.6	90.3
Aspartic acid	102.7	103.7	103.3	103.0	104.3	103.4
Threonine	98.7	97.4	97.0	98.2	98.0	97.9
Serine	99.4	98.7	98.3	100.4	98.7	99.0
Glutamic acid	99.0	99.2	98.6	98.0	98.6	98.7
Proline	97.4	98.2	99.0	99.3	98.5	98.5
Glycine	97.7	98.3	98.0	99.0	98.8	98.4
Alanine	97.7	99.3	100.0	98.3	98.8	98.8
Valine	97.7	99.5	98.7	98.2	97.5	98.3
Isoleucine	101.0	102.5	100.4	101.6	101.4	101.4
Leucine	101.3	102.0	100.4	100.8	101.7	101.2
Tyrosine	99.0	98.7	98.6	98.0	99.0	98.7
Phenylalanine	99.5	99.2	99.8	98.5	98.8	99.2

* = Average percentage of the five BS1, BS2, BS3, BS4 and BS5, also refer to Table 21 for corresponding concentration values determined from the autoanalyser.

TABLE 23

Quintiplate amino acid analysis of U.K. survey honey sample code No. 174 after ion-exchange chromatography

Amino acids	Concentration of amino acids (nMoles per g of honey)					
	A	B	C	D	E	Average*
Lysine	37	33	40	36	36	36
Aspartic acid	197	203	200	198	200	200
Threonine	150	154	146	152	146	150
Serine	355	348	350	358	348	352
Glutamic acid	412	410	416	414	410	412
Proline	6,610	6,586	6,620	6,640	6,600	6,610
Glycine	48	45	44	45	42	45
Alanine	165	167	180	175	173	172
Valine	125	120	114	123	120	120
Isoleucine	82	85	83	90	92	86
Leucine	56	60	58	60	65	60
Tyrosine	563	560	556	566	558	561
Phenylalanine	334	330	322	320	328	327
Weight of honey - 10.0 g						

* = Average concentration of the five A, B, C, D and E.

TABLE 24

Quintiplate amino acid analysis of U.K. survey honey sample code No. 174 plus 0.63 g of solution S1 after ion-exchange chromatography

Amino acids	Concentration of amino acids (nMoles per g of honey)					
	1	2	3	4	5	Average*
Lysine	142	130	136	134	145	137
Aspartic acid	317	320	313	310	308	314
Threonine	288	292	284	288	286	288
Serine	448	440	443	437	432	440
Glutamic acid	565	560	558	550	550	557
Proline	7,637	7,653	7,600	7,686	7,620	7.640
Glycine	221	223	218	226	218	211
Alanine	300	306	294	298	304	300
Valine	214	212	204	206	208	209
Isoleucine	195	200	195	200	193	197
Leucine	173	170	170	167	170	170
Tyrosine	718	718	716	712	710	715
Phenylalanine	548	546	542	540	544	544
Total weight of honey plus 0.63 g of solution S1 - 10.7 g.						

* = Average concentration of the five 1, 2, 3, 4 and 5.

TABLE 25

Quintiplate amino acid analysis of China light amber honey sample code No. 211 after ion-exchange chromatography

Amino acids	Concentration of amino acids (nMoles per g of honey)					
	A	B	C	D	E	Average*
Lysine**	-	-	-	-	-	-
Aspartic acid	170	176	174	170	175	173
Threonine	19	21	18	21	19	20
Serine	92	94	90	90	94	92
Glutamic acid	298	302	296	300	294	298
Proline	1,777	1,830	1,800	1,837	1,850	1,820
Glycine	60	62	62	60	58	60
Alanine	86	88	90	90	87	88
Valine	35	34	38	33	35	35
Isoleucine	63	60	62	60	64	62
Leucine	82	78	80	83	80	80
Tyrosine	105	108	106	107	110	107
Phenylalanine	535	537	530	524	525	530
Weight of honey - 10.0 g.						

* = Average concentration of the five A, E, C, D and E.

** = No value for lysine was recorded. This could be because lysine, always low, was in fact below the limit of detection or that the very large ammonia peak had overshadowed lysine and other basic amino acids. These amino acids occurred infrequently and were difficult to resolve on the autoanalyser for reason stated. However, the paper electrophoresis and chromatography combination did so resolve these basic amino acids but only trace amounts (1 nanomole) were present.

TABLE 26

Quintiplate amino acid analysis of China light amber honey sample code No. 211 plus 0.65 g of solution S1 after ion-exchange chromatography

Amino acids	Concentration of amino acids (nMoles per g of honey)					
	1	2	3	4	5	Average*
Lysine	108	107	118	113	107	110
Aspartic acid	286	290	295	283	290	290
Threonine	172	175	166	172	170	171
Serine	198	200	203	204	195	200
Glutamic acid	450	468	462	456	454	458
Proline	3,256	3,197	3,183	3,142	3,250	3,206
Glycine	248	250	242	245	240	245
Alanine	230	228	222	228	223	226
Valine	132	128	130	134	130	130
Isoleucine	180	175	184	178	178	180
Leucine	195	197	194	194	190	194
Tyrosine	303	296	297	307	294	300
Phenylalanine	750	744	743	737	740	743
Total weight of honey plus 0.65 g of solution S1 - 10.7 g.						

* = Average concentration of the five 1, 2, 3, 4 and 5.

TABLE 27

Quintiplate amino acid analysis of Mexico Yuctan honey sample code No. 227 after ion-exchange chromatography

Amino acids	Concentration of amino acids (nMoles per g of honey)					
	A	B	C	D	E	Average*
Lysine**	-	-	-	-	-	-
Aspartic acid	414	422	420	425	420	420
Threonine	230	220	225	228	222	225
Serine	204	200	207	198	200	202
Glutamic acid	1,256	1,264	1,183	1.173	1.190	1.213
Proline	4,512	4,518	4,445	4.504	4,478	4,490
Glycine	68	66	65	66	65	66
Alanine	230	236	232	227	224	230
Valine	115	112	116	118	110	114
Isoleucine	80	78	80	75	72	77
Leucine	64	60	62	62	58	61
Tyrosine	180	173	177	177	176	177
Phenylalanine	1,684	1,643	1,652	1,668	1,636	1,657
Weight of honey - 10.0 g						

* = Average concentration of the five A, B, C, D and E.

** = Analysis of lysine difficulties, refer to Table 25.

TABLE 28

Quintiplate amino acid analysis of Mexico Yuctan honey samples code No. 227 plus 0.63 g of solution S2 after ion-exchange chromatography

Amino acids	Concentration of amino acids (nMoles per g of honey)					
	1	2	3	4	5	Average*
Lysine	100	106	96	94	108	101
Aspartic acid	516	518	522	514	528	520
Threonine	360	354	358	364	362	360
Serine	307	300	295	306	302	302
Glutamic acid	1,287	1,317	1,323	1,315	1,332	1,315
Proline	5,720	5,665	5,672	5,716	5,647	5,685
Glycine	240	238	242	244	247	242
Alanine	364	358	352	356	354	357
Valine	204	202	208	200	206	204
Isoleucine	186	196	190	192	186	190
Leucine	166	177	175	173	168	172
Tyrosine	363	360	353	350	357	357
Phenylalanine	1,788	1,810	1,762	1,800	1,840	1,800
Total weight of honey plus 0.63 g of solution S1 - 10.63 g						

* = Average concentration of the five 1, 2, 3, 4 and 5.

TABLE 29

Recoveries of amino acids in solution S1 after mixing with honey samples: U.K. survey code No. 174, China light amber code No. 211 and Mexico Yuctan code No. 227

Amino acid	Percentage recoveries of amino acid* (%)		
	UK	CLA	MY
Lysine**	89.2	92.0	96.6
Aspartic acid	101.6	101.1	100.4
Threonine	100.1	99.4	99.8
Serine	99.2	98.8	99.4
Glutamic acid	98.0	99.1	98.5
Proline	100.2	99.9	99.4
Glycine	97.8	100.6	98.2
Alanine	99.8	99.9	97.7
Valine	101.6	98.9	100.9
Isoleucine	99.8	101.3	99.7
Tyrasine	100.1	101.3	98.9
Phenylalanine	99.5	100.2	100.0

* The percentage values obtained were calculated by using the concentration values given in Tables 23, 25 and 27 with those given in Tables 24, 26 and 28, respectively. The following equation was utilized

$$\text{Percentage recovery of amino acid} = \frac{HS \times IA - H \times I}{S1} \times 100$$

where HS = the total weight of honey plus the amount of solution S1 added, refer to footnotes to Tables 24, 26 and 28 for weight values used for each honey sample.

IA = individual average amino acid concentration value in each honey sample to which solution S1 was added, refer to Tables 24, 26 and 28.

H = the weight of honey used, refer to Tables 23, 25 and 27 for weight values used for each honey sample.

I = individual average amino acid concentration value in each honey, refer to Tables 23, 25 and 27.

S1 = the concentration of each amino acid calculated in the amount of solution S1 added to each honey sample from those given in Table 5.

** Analysis of lysine difficulties, refer to Table 25.

values greater than 500 nmoles per g of honey.

Replicate recovery analysis agreed to within ±1% to ±5% of the average values.

Mellissopalynology

The pollen content of a honey sample was determined microscopically as described. The pollen grains which were identified usually at both plant family and genus level as detailed in Table 7 and Appendix VIII. The pollen grains were then counted and presented in terms of frequency class for each honey sample as described. The data and characteristics from the predominant and secondary frequency classes of pollen grains was utilised for statistical analysis and were numerically coded as required by the computer software package - Statistical Package for Social Sciences for ease of differentiation between different grains. The numerical codes together with the botanical names, English names and plant family for a partial list of predominant and secondary pollen grains have been given in Table 30, and for the complete list of identified pollen grains are given in Appendix VIII. Some of the identified pollen grains were photographed at X400 magnification and these have been shown in Figure 8.

Amino Acids in the United Kingdom Samples Arranged in Order of Pollen Types

After evaluation and subsequent classification of the pollen grains into frequency classes for each sample of honey, the one hundred and ninty two honey samples of the United Kingdom survey were grouped into four pollen groups. These were: the predominant, secondary, secondary multiple and the unidentified type.

TABLE 30

List of pollen grains identified in honey and the numerical codes allocated to the pollen grains

Code No.	Botanical name[1] of Genus*	English name[1] of species	Plant family[1]
301	*Brassica*	Oilseed Rape	Cruciferae
302	*Hypericum*	Rose of Sharon	Guttiferae
303	*Tilia*	Silver Pendent Lime	Tiliaceae
304	*Impatiens*	Policeman's Helmet	Balsaminaceae
305	*Aesculus*	Horse Chestnut	Hippocastanaceae
306	*Ilex*	Holly	Aquifoliaceae
307	*Trifolium pratense*	Red Clover	Leguminosae
308	*Trifolium repens*	White Clover	"
309	*Vicia*	Broad or Field Bean	"
310	*Rubus*	Blackberry	Rosaceae
311	*Prunus/pyrus*	Plum/pear	"
313	*Epilobium*	Rosebay Willowherb	Onagraceae
314	*Anthriscus*	Cow Parsley	Umbelliferae
315	*Heracleum*	Hogweed	"
316	*Urtica*	Common Nettle	Urticaceae
317	*Corylus*	Hazel	Corylaceae
318	*Castanea*	Sweet Chestnut	Fagaceae
319	*Calluna*	Ling	Ericaceae
320	*Ligustrum*	Privet	Oleaceae
321	*Teucrium*	Wood Sage	Labiatae
322	*Achillea*	Millfoil	Compositae
323	*Carduus*	Welted Thistle	"
324	*Taraxacum*	Dandelion	"
325	*Lotus*	Birdsfood Trefoil	Leguminosae
326	*Clematis*	Traveller's Joy	Ranunculaceae
327	*Cotoneaster*	Cotoneaster	Rosaceae
328	*Myosotis*	Forget-me-not	Boraginaceae
329	*Linaria*	Common Toadfax	Scrophulariacea
330	*Alnus*	Alder	Betulaceae
331	*Banksia*	Honeysuckle**	Proteaceae
332	*Eucalyptus*	Aromatic Gum	Myrtaceae
333	*Fagopyrum*	Buckwheat	Polygonaceae
334	*Echium*	Viper's Bugloss	Boraginaceae
335	*Eucalyptus*	Eucalyptus	Myrtaceae
336	*Helianthus*	Sunflower	Compositae
337	*Melilotus*	Yellow Melilot	Leguminosae
338	*Onobrychis*	Sainfoin	"
339	*Robinia*	False Acacia	"
340	*Salix*	Willow (Sallow)	Salicaceae
341	*Viguiera*	'Tah' Railway Daisy	Compositae
342	*Myrtaceae*	Eucalyptus	Myrtaceae
343	*Helleborus*	Christmas Rose	Ranunculaceae
344	*Fragaria*	Garden Strawberry	Rosaceae

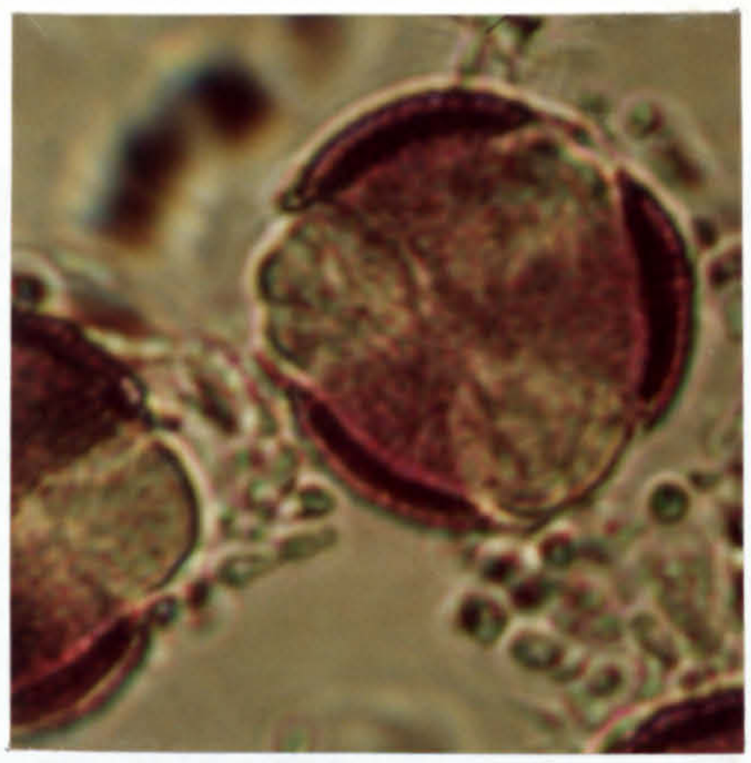
1 - These are according to Sawyer (1981) and Crane, Walker and Day (1984).

* - Due to difficulty of pollen identification of some plants especially of the genus *Brassica* for example, which is detailed in Table 7 and also Appendix VIII, only the genus latin name is given under Botanical column and the likely species to be found in honey is given under English column.

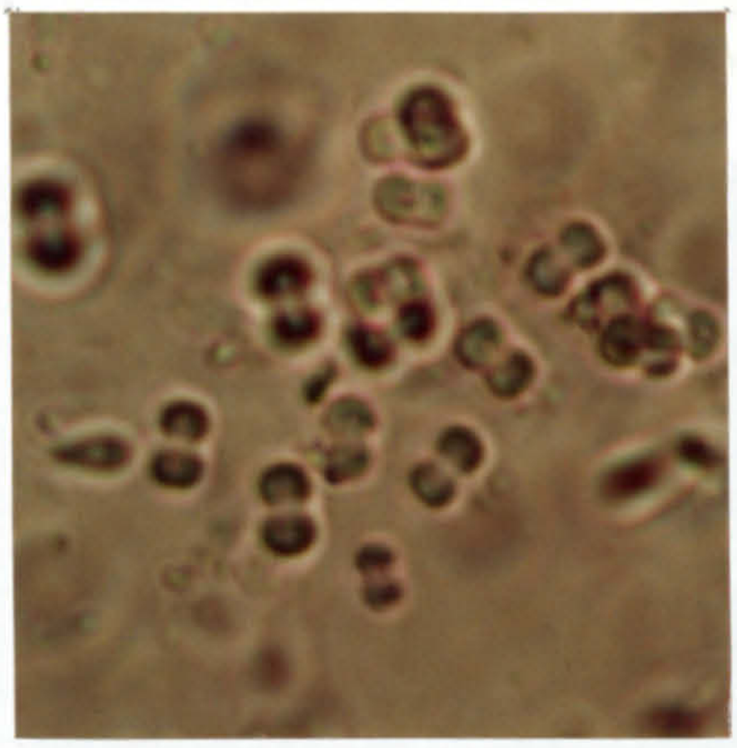
** - Australian For complete list of all the pollens identified in all the two hundred and fifty six samples examined, refer to Appendix VIII.

FIGURE 8

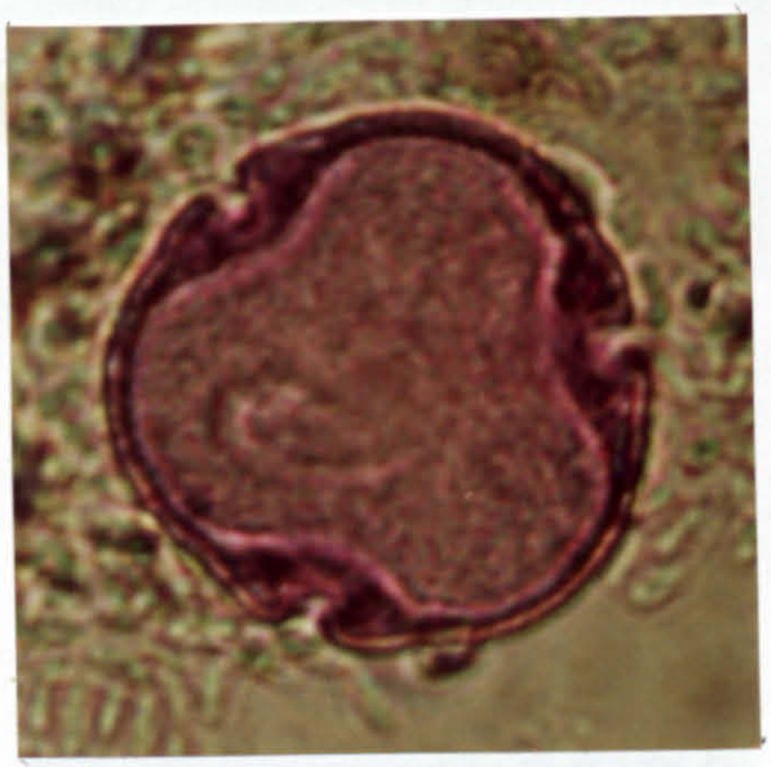
Microscopical identification of pollens present in the U.K. survey and foreign and commercial honey



Brassica
U.K. Code No. 27

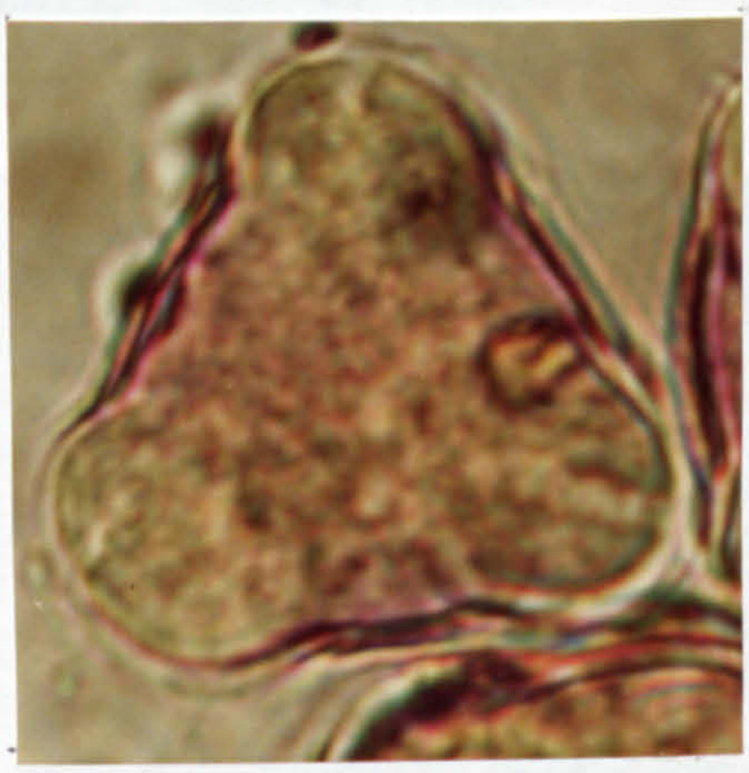


Myosotis
U.K. Code No. 106



Tilia
U.K. Code No. 23

Ligustrum
English Code No. 250

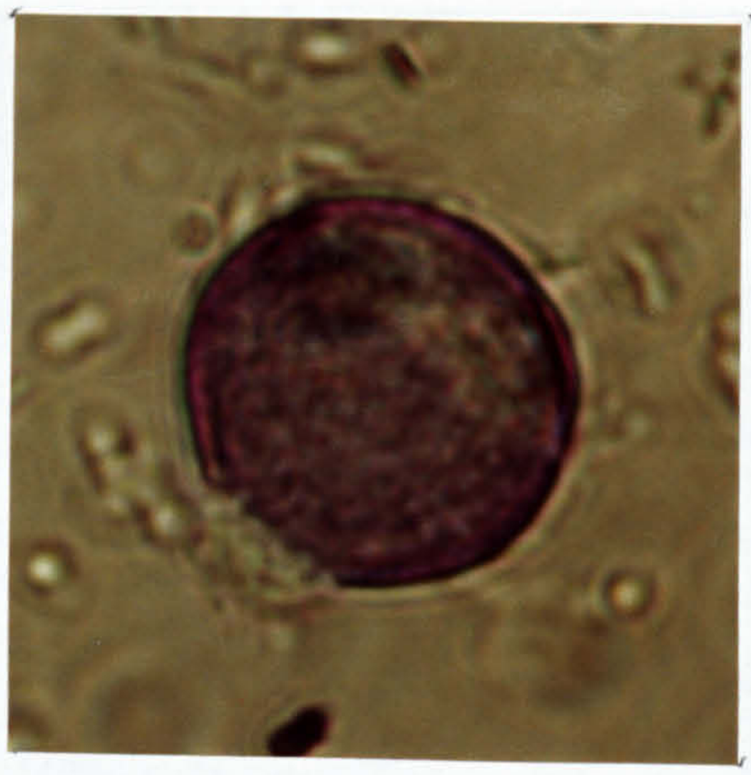


Rubus
U.K. Code No. 105

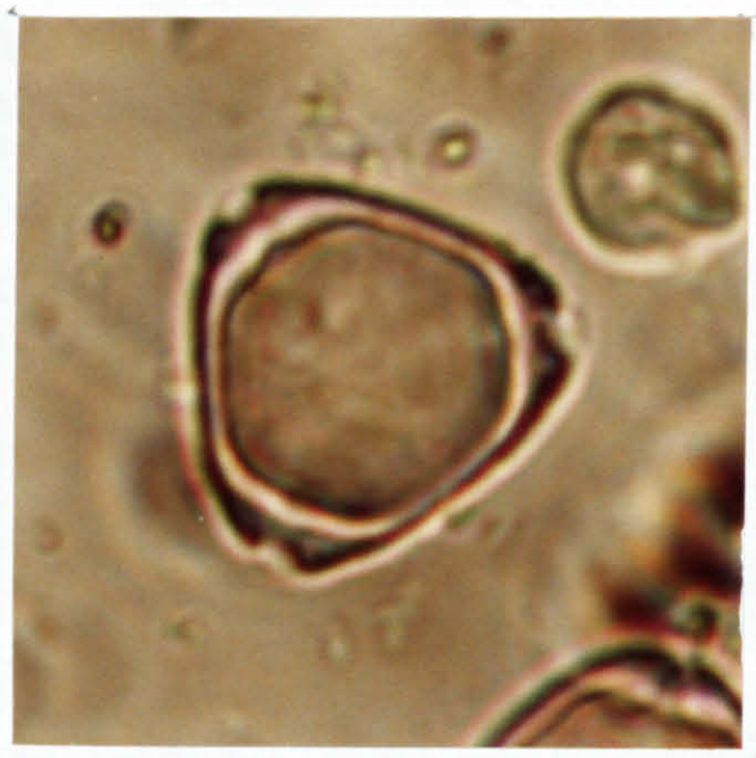
Castanea
U.K. Code No. 13

25 µm

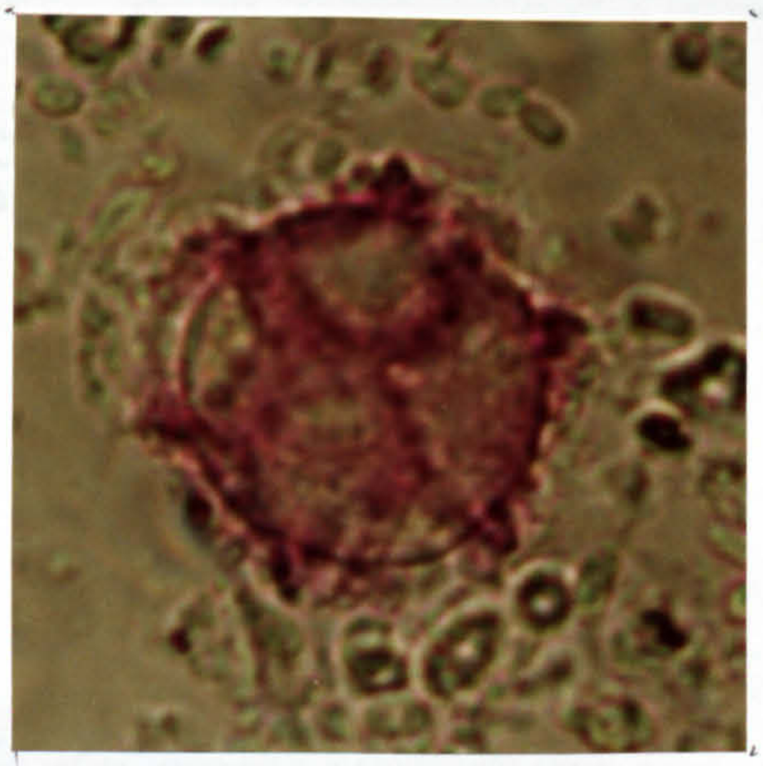
Figure 8 cont'd....



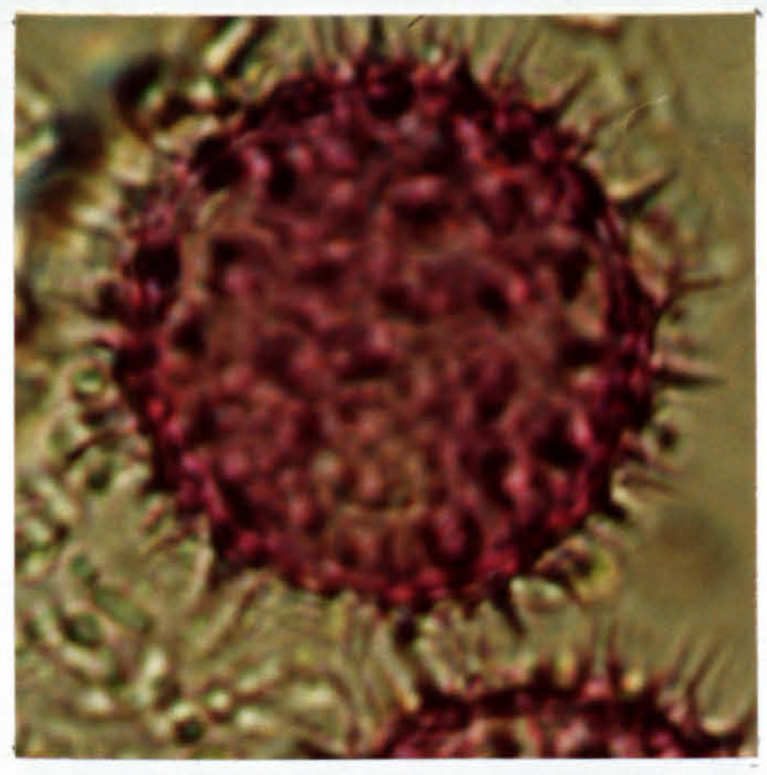
Melilotus
Canada Code No. 203



Eucalyptus
Australia Code No. 197



Taraxacum
China Code No. 208

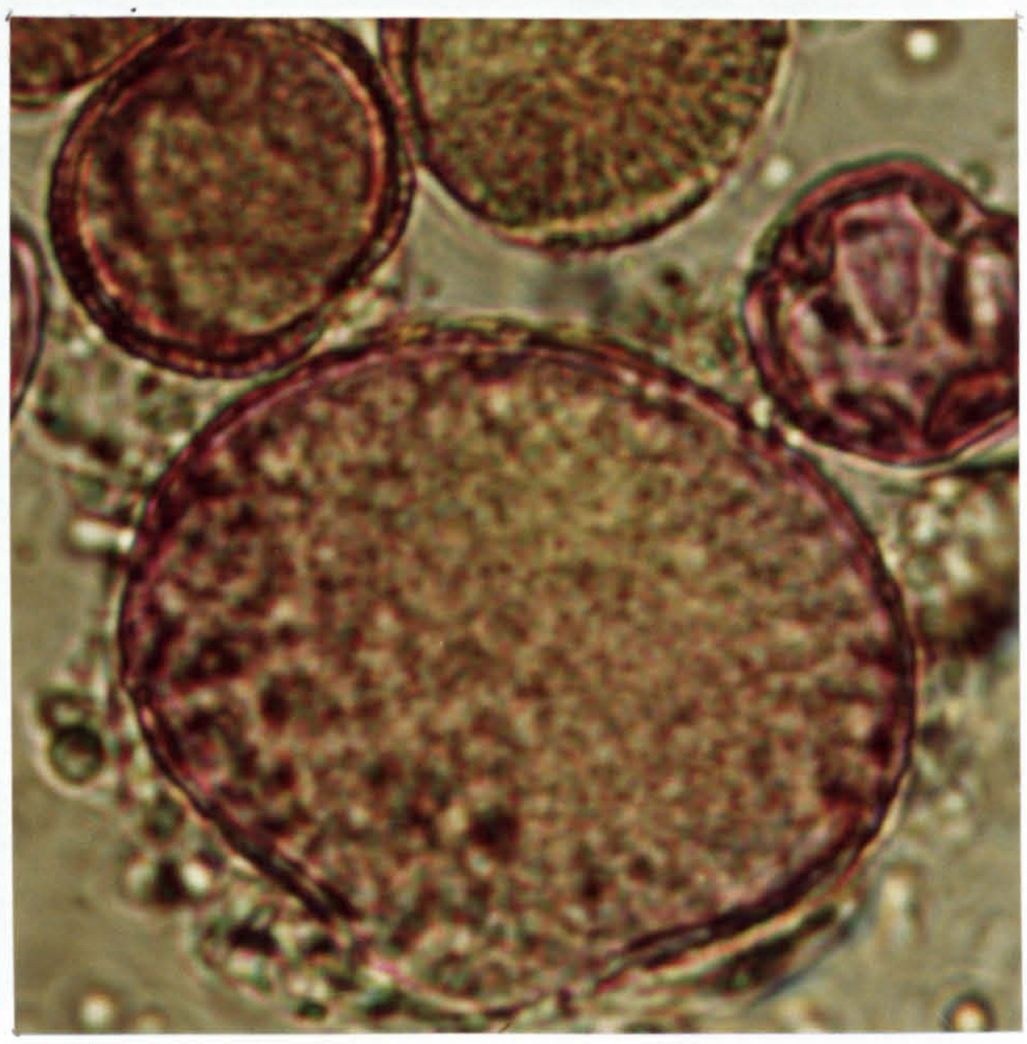


Helianthus
France Code No. 218

Echium
Yellow Box Code No. 246

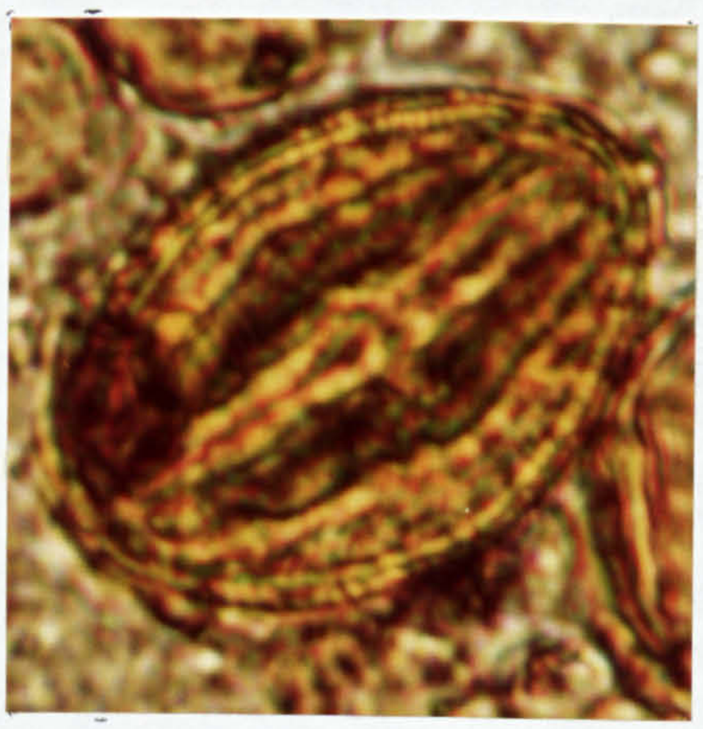
Loranthus
Australia Code No. 197

Figure 8 cont'd....



Trifolium repens
English Code No. 248

Pyrus
U.K. Code No. 120



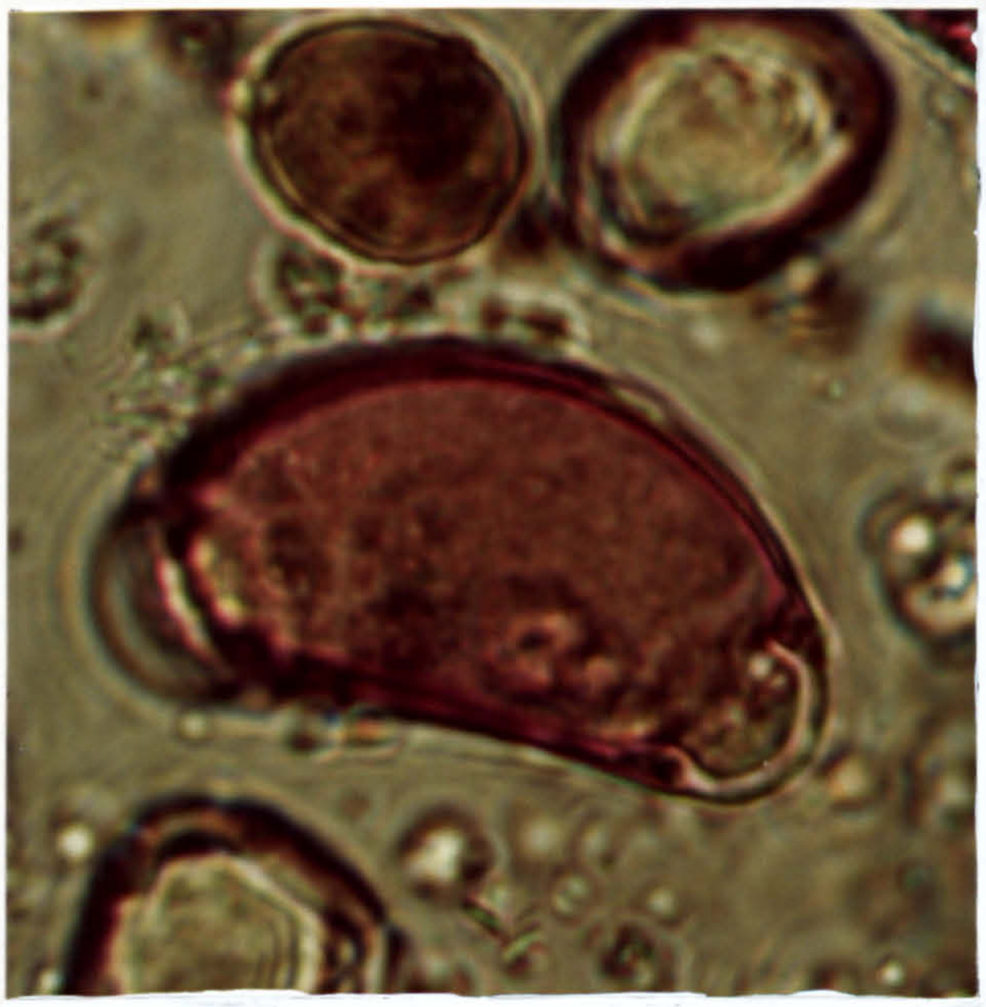
Fagopyrum
China Code No. 209

Viquiera
Cayman Island Code No. 207

Figure 8 cont'd....

Calluna
English Code No. 251

Acacia
Australia Code No. 195



Banksia
Australia Code No. 194

Vicia
U.K. Code No. 12

25 µm

Predominant pollen type

Honey samples containing the predominant frequency class pollen, that is, those containing over 45% pollen were allocated to this category. There were one hundred and thirteen honey samples that accounted for nine predominant floral pollens. Pollens from five major floral sources *Brassica*, *Trifolium repens*, *Castanea*, *Calluna* and *Myosotis* were responsible for one hundred and six honey samples.

The honey samples in each of the five pollen groups were arranged in a decreasing order of pollen percentages. The pollen content and the response to questionnaire question 4, for each sample belonging to these five named *Brassica*, *Trifolium repens*, *Castanea*, *Calluna* and *Myosotis* pollen groups have been lised in the order of Tables 31, 34, 37, 40 and 43. The amino acid concentration measurements and the responses to questionnaire questions 3, 5 and 6 have been given in the order of Tables 32, 35, 38, 41 and 44.

From the amino acid maps similar to that shown in Figure 7 the following ninhydrin positive substances were identified using reference standards in each honey sample within each of the five pollen groups. These ninhydrin positive substances were: α- and β-alanine, α- and γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, glucosamine, glycine, glutamine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, methyl-histidine, phenylalanine, pipecolic acid, proline, serine, threonine, tryptophan, tyrosine, valine and the two unidentified spots labelled as 'x' and 'p'. Differences in occurrence of ninhydrin positive substances were observed on the amino acid maps and these were evaluated within and between each of the five pollen groups. For reasons of clarity of presentation it was

(next on page 162)

KEY TO TABLE 31

In the key listed below, notes 1, 2, 3 and 4 are common to all the pollen tables of the U.K. survey honeys, that is, Tables 31, 34, 37, 40, 46, 49 and 52.

1. For questionnaire number and approximate geographical location in the United Kingdom, refer to appendix I (exact location of apairy not given to maintain confidentiality).
2. The percent pollen quoted is the numerical value of the number of pollen grains of an identified floral source multiplied by the number one hundred and then divided by the total number of polyfloral pollen grains counted. Thus, the pollen percentage values for each floral source in each honey sample was grouped into the appropriate following frequency classes:

   Predominant pollen (>45%)
   Secondary pollen (16% - 45%)
   Important Minor pollen (3% - 15%)
   Minor pollen (>13%)
3. Genus quoted only. In Appendix VIII are given the common English names for the likely species and also the plant family and genus are quoted therein.
4. RQQNo4 - response to questionnaire question No. 4 - these were:

OR	=	oilseed rape	G	=	Gooseberry
C	=	Clover	Pr	=	Pears
NS	=	Not specified	Pm	=	Plums
S	=	Sycamore	D	=	Dandelion
L	=	Lime	CN	=	Chestnut (horse or sweet chestnut not specified)
B	=	Blackberry	W	=	Willows
F	=	Fruit (apple, pears, plums, etc)	Py	=	Pyrus
A	=	Apple	SF	=	Soft fruit (peach, pear, etc)
GF	=	Garden flowers (Roses, Dandelions, etc)	LG	=	Local gardens
Sn	=	Sainfoin	Pv	=	Privet
Bn	=	Beans	GR	=	Golden rod
HD	=	Honeydew	Bs	=	Blossom
WC	=	White Clover	Ch	=	Cherry
Mx	=	Mixed (whether flowers, shrubs or trees not specified)	Rw	=	Ragwort
SB	=	Spring blossom	LBs	=	Late beanstick
H	=	Hawthorn	Rbw	=	Rose Bay Willow
Bb	=	Bulbs	Hz	=	Hazel
DN	=	Dead Nettle	Ce	=	Catoneaster
FB	=	Field Bean	G	=	Gardens
Hr	=	Heather (ling, gorse, broom, clover billberry)	FW	=	Firwood
Wh	=	Willowherb (Rosebay)	FT	=	Field Thistle
Br	=	Bracken	BH	=	Bell Heather
WB	=	Water balsam	Sh	=	Sea Lavender
Bm	=	Bramble	WS	=	Wood sage
Hw	=	Hedgerow (type and species not specified)	Bu	=	Bugoss
P	=	Pasture	Tr	=	Tree (Elm, Birch, Marple, etc)
Bl	=	Balsam	CV	=	Calluna vulgaris
E	=	Eucalyptus	SpF	=	Spring flowers
Lg	=	Ling	Rb	=	Rowanberry
Fb	=	Fruit blossom	Ia	=	Industrial area
HC	=	Horse Chestnut	UF	=	Urban flora (not specified)
Sh	=	Shrubs (not specified)			

Key to Table 31 Cont'd

The responses listed on the previous page were those quoted by the participating beekeeper. Some of the responses were vague, ambiguous and unclear in definition, in that, the type and species of plants, shrubs and trees were not specified. In these cases it was assumed that the quoted responses were those which one would generally associate with the common known plant, shrubs and trees of the U.K. (some examples have been given in parenthases).

A full list of all floral genera that have been observed in the samples examined are given in Appendix VIII. For reasons of clarity in presentation it was necessary in Tables 31 - 60 to omit column entries where there is no pollen representation of the species concerned.

TABLE 31

Frequency of distribution of pollen grains in samples of *Brassica* honeys (ex. U.K. survey)

Floral genus	Pollen (%)[2,3]														
	Honey sample code No[1]														
	27	108	58	56	61	26	64	180	66	8	153	155	150	151	67
Acer										0.7					
Brassica	92.5	89.0	88.7	88.0	88.0	87.0	86.4	86.3	85.8	84.7	84.7	84.1	83.4	82.6	82.2
Castanea															
Heracleum		0.3			0.9										
Hypericum															
Ligustrum			0.1												
Onobrychis															
Pinus															
Prunus/Pyrus		1.7	4.1	1.7	2.2	3.0		3.8	4.7	2.4	0.2	5.8	6.2	4.9	4.4
Rubus	0.7	2.0		1.1		1.0			0.3	1.2					
Taraxacum															
Trifolium pratense					1.2		0.6								
Trifolium repens				0.8			1.1								
Urtica															
Vicia											6.7				
Shrivelled pollen	6.0	5.2	5.7	6.5	4.6	7.0	11.5	7.1	7.5	3.0	5.7	6.1	6.5	8.0	11.8
Unidentified pollen	0.8	1.7	1.2	1.7	3.0	1.7	0.9	2.7	1.7	8.0	2.6	4.1	3.8	4.5	1.5
Total pollen grains counted	548	345	577	460	408	756	339	337	359	419	387	295	337	288	338
RQQNo4[4]	OR	OR	OR	OR	OR	OR	OR	OR	OR,S	OR	NS	NS	C	NS	S,OR C,L, B

The notes to key 1, 2, 3 and 4 are given on the key sheet.

Table 31 cont'd....

Floral genus	Pollen (%)[2,3] Honey sample code No[1]														
	182	140	135	54	68	143	118	138	137	132	145	124	114	115	175
Acer															
Brassica	81.6	81.3	80.3	80.0	79.3	78.7	78.1	77.6	77.3	75.4	74.4	73.9	73.0	72.8	67.5
Castanea							2.2								
Heracleum															
Hypericum															
Ligustrum															
Onobrychis															
Pinus															
Prunus/pyrus	5.1	2.7	3.8	10.5	6.9	4.0	6.4	2.9	0.9		16.7		11.3	0.9	4.4
Rubus					0.4					7.6		6.9			2.8
Taraxacum					0.4										
Trifolium pratense					0.4										
Trifolium repens	2.2					2.9									
Urtica															
Vicia								0.9		4.7		3.5		1.2	
Shrivelled pollen	6.8	11.3	11.0	7.7	10.0	9.9	9.7	14.8	16.4	10.4	4.9	8.7	10.3	20.3	14.2
Unidentified pollen	4.2	4.7	4.8	1.8	2.3	4.4	3.5	3.8	5.3	1.9	3.9	6.9	5.3	4.7	11.0
Total pollen grains counted	354	257	290	506	261	272	310	344	225	317	305	230	300	320	317
RQQNo4[4]	OR	OR	OR	OR	OR	F,S, OR	A,G, Pr,Pm D	OR	OR	OR	OR	OR,CN, S,W,A	S,Py OR	OR	OR

Table 31 cont'd....

Floral genus	Pollen (%)[2,3] Honey sample code No[1]													
	92	88	51	188	162	74	11	42	131	144	110	185	179	87
Acer					5.3									
Brassica	66.9	66.0	64.5	63.5	60.3	56.1	56.0	55.2	54.1	53.0	51.2	47.9	45.2	45.1
Castanea			1.8			4.6								
Heracleum										2.4			3.0	
Hypericum							2.1							
Ligustrum										5.8				
Onobrychis								11.3						
Pinus						0.4								
Prunus/pyrus	10.0			2.4		11.4	28.9	4.0		1.8		20.1	6.8	
Rubus	3.8	3.2	26.9			5.9				0.6	17.8			3.2
Taraxacum						2.5								
Trifolium pratense														
Trifolium repens					5.3			1.3	3.4					
Urtica									4.4					26.0
Vicia	2.7	17.5		3.7		0.8			18.4		11.7	13.4	3.0	5.4
Shrivelled pollen	11.4	11.3	6.4	23.2	22.5	15.6	7.1	23.0	14.6	20.4	13.7	12.1	23.8	17.3
Unidentified pollen	5.2	2.0	1.8	7.2	6.5	2.5	5.7	5.2	5.1	16.0	5.5	6.4	18.2	3.0
Total pollen grains counted	290	406	482	293	262	237	421	221	294	328	324	313	369	466
RQQNo4[4]	OR,S	OR	OR	B	S	C,L, SF,GF	OR	Sn	Bn,HD, Wc, Mx	L	SB,S, H,F, BD,DN	NS	UN,B R,L,C	FB

TABLE 32

Free amino acids in *Brassica* honey

Amino Acids	Concentration (nMoles per g of honey)														
CNo[1]	27	108	58	56	61	26	64	108	66	8	153	155	150	151	67
Lys	133	42	109	161	142	147	141	60	112	263	110	327	101	139	21
Asp	65	43	66	64	65	67	134	95	63	62	57	142	80	64	62
Thr	95	32	64	22	68	102	226	33	41	73	19	41	24	30	52
Ser	61	20	75	73	68	85	314	55	51	63	41	88	62	66	55
Glu	94	73	120	112	117	148	361	136	72	93	96	171	88	78	101
Pro	1570	1570	1960	1350	2180	2010	1490	1550	1280	1880	1120	1390	1610	1370	1600
Gly	34	19	37	23	50	48	58	50	84	33	32	56	32	24	39
Ala	79	26	82	44	132	81	192	57	70	61	42	75	60	31	81
Val	45	21	49	56	51	59	113	35	T	37	30	44	30	T	61
Ile	33	16	32	55	45	45	75	36	38	27	35	72	32	27	43
Leu	37	56	32	48	34	60	70	36	27	40	28	54	36	30	39
Tyr	42	28	60	51	65	75	36	67	35	61	24	46	58	34	42
Phe	183	54	54	139	155	386	129	123	102	56	56	135	87	84	114
Sample[2] Date	6/82	6/82	6/81	5/82	6/82	6/82	7/81	6/81	6/82	6/81	Un	Un	Un	Un	8/82
Heating[3]	No	No	Yes	No	No	No	No	Yes	Yes	No	Un	Un	Un	Un	Yes
Sugar[4] Feeding	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Un	Un	Un	Un	No

1. CNo = Code No, refer to Appendix 1 for questionnaire number.
2. Sample Date = Date sample was taken from the hive: Question number 3, is given as month/year.
3. Heating = Sample heated or otherwise: Question number 5.
4. Sugar Feeding = Were the bees fed sugar or otherwise: Question number 6.

T = Trace amounts.

Un = Not specified on the questionnaire.

This key is common to Tables 35, 38, 41, 44, 47, 50, 53 and 55.

Table 32 cont'd.....

Amino Acids	Concentration (nMoles per g of honey)														
	CNo[1] 182	140	135	54	68	143	118	138	137	132	145	124	114	115	175
Lys	66	126	T	63	100	221	78	91	46	72	100	88	48	48	87
Asp	72	83	87	69	94	102	81	88	125	111	85	85	117	288	156
Thr	20	37	36	20	252	36	79	34	20	16	18	34	37	42	64
Ser	44	52	70	38	47	80	99	49	55	46	55	85	76	57	141
Glu	112	165	203	111	161	161	210	115	142	162	127	133	154	115	194
Pro	1950	1910	1650	1830	1800	1460	4060	1280	1340	1270	1010	1580	1650	1720	2320
Gly	45	30	88	47	43	50	47	30	22	26	46	28	43	41	63
Ala	52	63	72	48	51	72	126	57	60	51	68	71	87	69	86
Val	T	55	43	31	38	70	81	33	T	32	39	83	52	49	51
Ile	34	62	59	29	32	63	78	42	27	29	40	40	39	45	48
Leu	25	60	45	31	43	44	66	35	30	23	31	24	46	33	42
Tyr	63	43	112	T	T	57	167	T	T	50	45	60	T	221	74
Phe	150	99	458	111	92	176	138	50	49	297	196	242	95	1080	358
Sample[2] Date	8/82	5/82	5/82	6/82	5/82	6/82	6/82	5/82	5/82	6/82	5/82	5/82	6/82	6/82	5/82
Heating[3]	No	No	No	Yes	Yes	No	Yes	No	Yes	No	No	No	No	No	No
Sugar[4] Feeding	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes

Table 32 cont'd.....

Amino Acids	Concentration (nMoles per g of honey)													
	CNo[1] → 92	88	51	118	162	74	11	42	131	144	110	185	179	87
Lys	118	161	128	129	150	97	112	359	110	266	61	123	181	160
Asp	46	125	62	67	109	72	62	96	117	71	71	116	83	113
Thr	23	37	117	42	83	85	55	284	53	45	71	96	50	68
Ser	42	72	94	50	72	75	47	75	56	75	62	107	57	95
Glu	99	170	128	134	141	135	79	116	190	167	153	262	165	247
Pro	1630	1760	2170	1590	1970	2520	1160	1890	3480	3960	1480	4630	3680	4760
Gly	35	56	58	47	44	37	28	35	45	58	31	97	56	64
Ala	49	71	86	66	83	94	38	60	70	81	67	130	81	97
Val	29	36	75	35	63	60	34	48	41	56	43	99	61	46
Ile	28	44	41	31	58	80	25	50	31	47	34	71	45	36
Leu	29	39	44	20	81	70	20	54	23	41	22	58	43	24
Tyr	T	24	61	T	71	120	46	170	48	35	58	74	78	T
Phe	63	122	73	55	67	337	75	373	107	179	190	210	137	100
Sample[2] Date	6/82	5/82	5/82	6/81	6/82	7/81	6/81	8/81	8/82	10/82	5/82	Un	8/81	6/82
Heating[3]	No	No	No	No	No	No	No	No	Yes	No	No	Un	Yes	No
Sugar[4] Feeding	Yes	No	No	Yes	Yes	No	Yes	Yes	No	No	No	Un	Yes	No

TABLE 33

Occurrence of ninhydrin positive substances in *Brassica* honeys

Ninhydrin positive substance	Sample numbers in which given compound was found not* to occur
β-Ala	8, 58, 61, 64, 67, 74, 87, 124, 143
α-Abu γ-Abu	67, 118, 153
Arg	54, 56, 58, 66, 88, 110, 114, 115, 118, 124
Asn	8, 11, 27, 42, 56, 58, 61, 64, 66, 92, 108, 110, 115, 180
Cys	8, 11, 58, 67, 74, 118, 124, 132
Glu	8, 11, 26, 27, 67, 88, 92, 108, 124, 135, 175, 179, 180
His	88
Ile Leu	132
Me-His	26, 58, 66, 67, 74, 92, 124, 140
Trp	8, 11, 67, 88, 92, 114, 138, 140, 151, 153, 179, 182, 188
'x'	8, 27, 51, 54, 56, 58, 64, 67, 68, 74, 87, 92, 110, 114, 140, 162

In addition to the other amino acids found in all samples, the following ninhydrin positive substances were also present. These were: hydroxyproline in sample numbers 74, 109, 114 and 124; pipecolic acid in sample numbers 42, 61 and 64 and the unidentified spot 'p' in sample numbers 27, 66, 68, 114 and 162.

* = The presentation of every amino acid present in all the samples would give an excessively long list which was considered not justifiable. However, unless stated otherwise the following common amino acids were found in all samples: lysine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine and phenylalanine. Small amounts of amino acids were often detected by the sensitive paper electrophoresis and chromatography technique used and not by the amino acid analyser. These conditions apply to Tables 36, 39, 42, 45, 48, 51, 54, 56, 59 and 62.

FIGURE 9

Amino acid map obtained from honey U.K. sample No. 91 (*Brassica*).

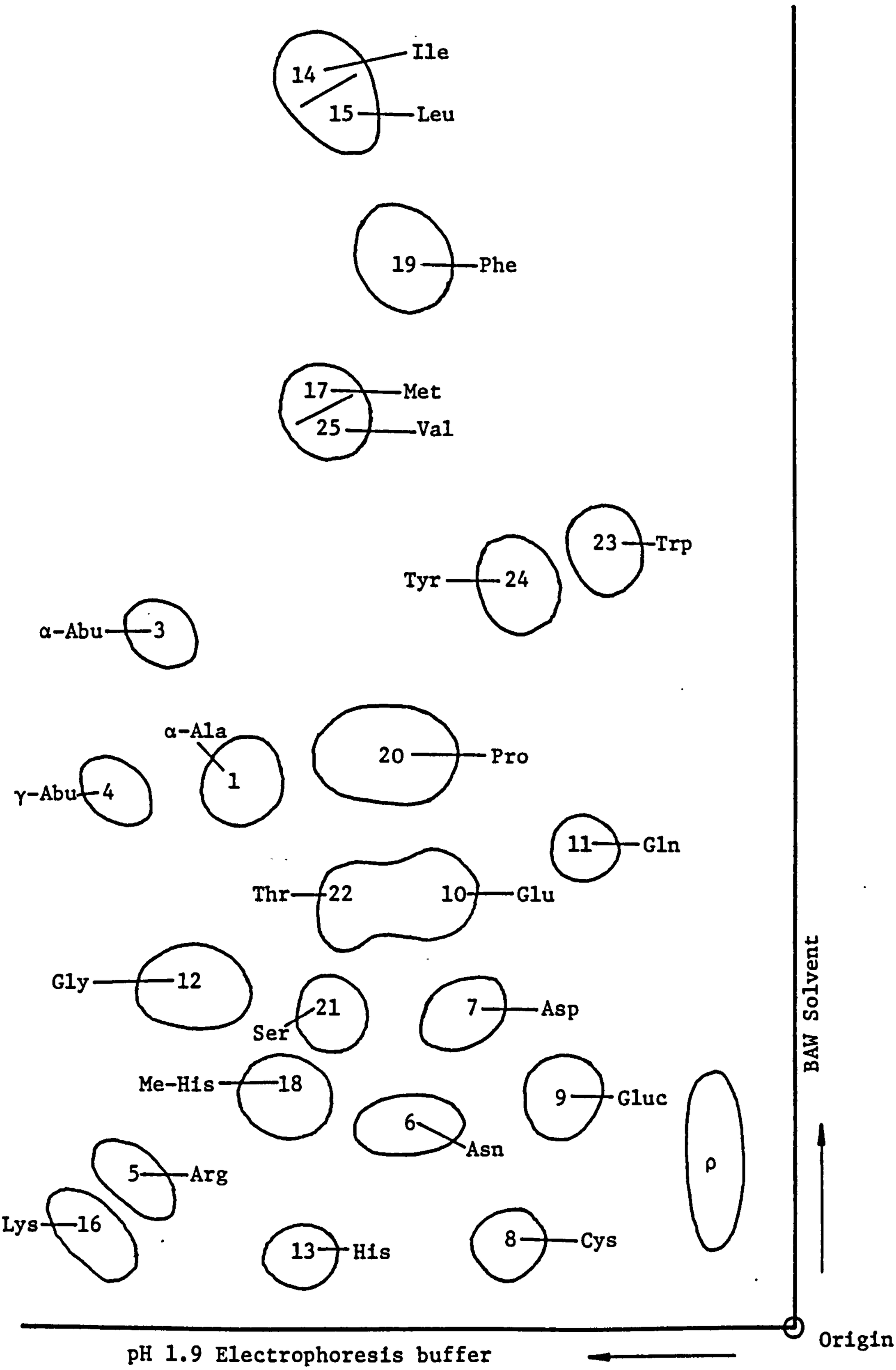


FIGURE 10

Amino acid map obtained from honey U.K. sample No. 131 - *Brassica*

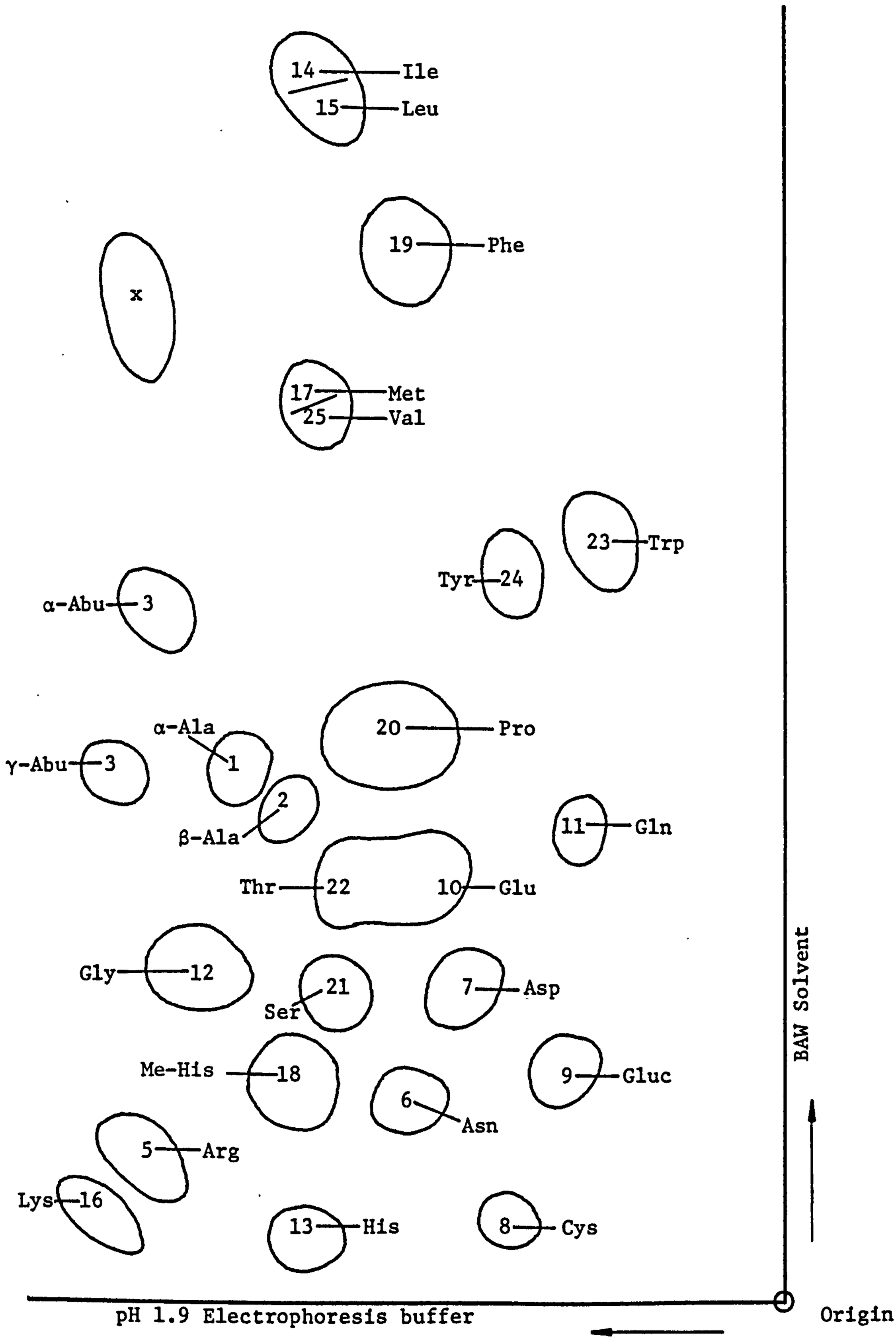


TABLE 34

Frequency of distribution of pollen grains in samples of *Trifolium repens* honeys (ex. U.K. survey)

Floral genus	Pollen (%)[2,3] Honey sample code No[1]														
	142	47	102	141	158	186	157	82	164	81	184	174	45	44	160
Acer															
Aesculus															
Brassica						3.3			5.2	0.6				15.7	
Calluna			1.1				9.2	9.4					14.7	1.5	
Corduus		0.2													
Castanea			3.8	3.1		2.5					1.4	10.0			1.5
Cotoneaster															
Epilobium				0.3	0.3		0.2			2.3	2.1			0.6	
Heracleum		1.4						1.6		6.1					
Impatiens													1.3		3.1
Ligustrum			0.2												1.5
Phlox			0.8												
Prunus/pyrus			9.8			10.8						2.6		7.0	
Rubus		1.7		1.9						2.3					
Salix															
Taraxacum													1.3		
Tilia						0.7									
Trifolium repens	78.1	73.6	70.7	70.7	68.8	67.6	67.1	63.4	62.7	62.5	61.8	60.4	60.2	59.3	59.3
Vicia									1.2						
Viola	0.4														
Shrivelled pollen	11.4	11.5	20.1	18.8	16.6	19.2	12.5	22.8	25.1	22.7	18.6	18.3	22.7	14.0	29.4
Unidentified pollen	8.4	2.5	2.3	7.1	5.1	6.6	10.9	2.7	5.8	3.5	16.1	7.4	1.1	1.9	5.1
Total pollen grains counted	297	488	263	293	343	395	432	254	343	344	280	230	457	464	194
RQQNo4[4]	NS	Hr	C,B	A	B,Wh, C	NS	Hr,Br	Hr,C	B,Wh	S,C	Wh	C,L, B	H	S,C	Wh,B

For notes to key 1, 2, 3 and 4 refer to Table 31.

Table 34 cont'd....

Floral genus	Pollen (%)[2,3] Honey sample code No[1]													
	117	103	101	35	34	20	183	98	154	163	116	134	192	100
Acer										1.8				
Aesculus							9.7							
Brassica												5.8		
Calluna														
Carduus			0.7					1.8				3.2		
Castanea	2.0	8.9		5.0					8.3			5.8	8.8	
Cotoneaster								9.5						
Epilobium	0.3					0.7			4.3	1.4	0.3	0.6		
Heracleum		1.3	0.9			0.9			1.7	1.4				
Impatiens														
Ligustrum				1.3										
Phlox														
Prunus/pyrus	3.4	7.6				16.4	3.6			6.8	2.5	2.3		
Rubus	2.0		10.8	6.8	2.0	5.0		1.8	4.7		2.2			9.2
Salix							10.7							
Taraxacum														
Tilia						0.7								
Trifolium repens	58.6	55.9	52.0	52.9	51.4	51.3	48.6	47.1	47.0	46.4	46.3	45.9	45.9	45.7
Vicia			0.5						1.3				9.5	0.9
Viola														
Shrivelled pollen	30.1	24.6	31.6	32.9	45.0	18.7	20.1	33.2	28.7	30.9	45.4	31.9	24.6	39.7
Unidentified pollen	3.4	1.7	2.5	1.0	1.0	6.3	7.3	6.6	4.0	11.3	3.3	4.4	11.1	4.5
Total pollen grains counted	292	236	434	395	321	450	383	274	300	278	361	342	305	339
RQQNo4[4]	B	B,C, H,L	B,WB, Wh	B,C	Bm,C	Mx	W,D, C	Hw,P	NS	S,DR	B,C	C,Bm	L,C	NS

TABLE 35

Free amino acids in *Trifolium repens* honey

Amino Acids	Concentration (nMoles per g of honey)														
	CNo[1] 142	47	102	141	158	186	157	82	164	81	184	174	45	44	160
Lys	197	268	175	377	81	345	149	90	230	131	109	128	295	101	324
Asp	133	164	164	96	106	225	88	45	73	49	45	222	99	55	72
Thr	104	90	89	89	54	94	85	35	106	91	34	138	175	102	77
Ser	111	128	204	109	74	176	77	50	88	93	45	171	168	91	83
Glu	302	249	444	276	167	474	186	113	191	128	134	494	281	148	203
Pro	4320	4780	4780	2920	3910	3780	5100	2790	4860	4210	2980	6750	5530	3780	7720
Gly	56	79	88	61	64	88	73	24	70	42	50	184	82	54	78
Ala	140	205	208	107	85	191	101	81	147	122	58	195	232	97	131
Val	92	128	105	87	67	91	67	33	85	60	45	150	118	73	92
Ile	182	261	115	82	117	122	55	40	494	50	52	116	87	139	49
Leu	134	163	55	55	101	98	62	31	457	71	95	81	87	109	97
Try	100	89	794	69	59	48	70	70	85	76	133	790	90	74	54
Phe	209	169	650	115	777	147	171	241	330	265	570	580	306	149	165
Sample[2] Date	9/82	9/82	8/81	6/82	8/82	Un	11/82	9/81	8/82	7/81	12/82	8/82	9/82	8/82	8/82
Heating[3]	No	No	No	No	No	Un	No	No	No	No	No	No	No	No	No
Sugar[4] Feeding	No	No	Yes	Yes	Yes	Un	No	Yes	No	Yes	No	No	No	Yes	Yes

For notes to key 1, 2, 3, T and Un, refer to footnote to Table 32.

Table 35 cont'd....

Amino Acids	Concentration (nMoles per g of honey)													
CNo[1]	117	103	101	35	34	20	183	98	154	163	116	134	192	100
Lys	70	125	197	289	95	147	74	72	243	158	168	T	187	137
Asp	349	142	90	100	190	90	44	129	920	115	290	310	272	97
Thr	52	107	58	84	99	107	28	42	145	75	41	97	155	47
Ser	129	143	65	87	81	94	40	86	407	89	128	143	237	46
Glu	552	371	138	333	160	197	114	149	1580	218	630	570	690	126
Pro	4120	4880	2800	4100	3280	3070	2250	3160	3790	4320	2750	3890	5480	2930
Gly	88	64	46	68	55	73	42	61	173	94	79	106	125	29
Ala	136	181	98	136	150	137	51	169	430	125	159	148	249	97
Val	39	105	47	69	65	65	43	60	52	67	39	78	130	46
Ile	34	92	105	129	173	42	31	68	48	61	37	98	144	89
Leu	17	80	66	95	118	52	54	60	51	103	27	52	119	87
Tyr	66	278	113	2210	580	100	350	82	133	42	40	89	70	72
Phe	T	284	239	1250	410	122	820	126	166	108	79	98	303	206
Sample[2] Date	8/81	8/82	10/81	9/81	11/81	8/81	6/82	9/82	Un	7/82	8/81	8/81	8/81	10/81
Heating[3]	No	No	No	No	No	Yes	No	No	Un	No	No	No	Yes	No
Sugar[4] Feeding	Yes	Yes	Yes	No	No	Yes	No	Yes	Un	No	Yes	Yes	No	Yes

TABLE 36

Occurrence of ninhydrin positive substances in *Trifolium repens* honeys

Ninhydrin positive substance	Sample numbers in which given compound was found not* to occur
β-Ala	82, 100, 101, 102, 134
γ-Abu	158
Arg	100, 141, 157, 158, 160, 163, 184
Asu	45, 81, 82
Cys	158
Gluc	34, 35, 100, 117, 158, 160, 117
Glu	100, 158, 160
Gln	154
His	134
Phe	158
Trp	158, 160

In addition to the other amino acids found in all samples, the following ninhydrin positive substances were also present. These were: pipecolic acid in sample numbers 34, 44, 81, 98, 100, 102, 116, 121, 142, 163 and 164; the unidentified spot 'x' in sample numbers 47, 134, 141, 160, 174, 175, 184 and 186 and unidentified spot 'p' in sample numbers 100, 101, 163 and 164. One other unidentified spot 'ε' was present in sample numbers 44 and 164.

* = Details as per Table 33.

FIGURE 11

Amino acid map of U.K. survey No. 44 - *Trifolium repens*.

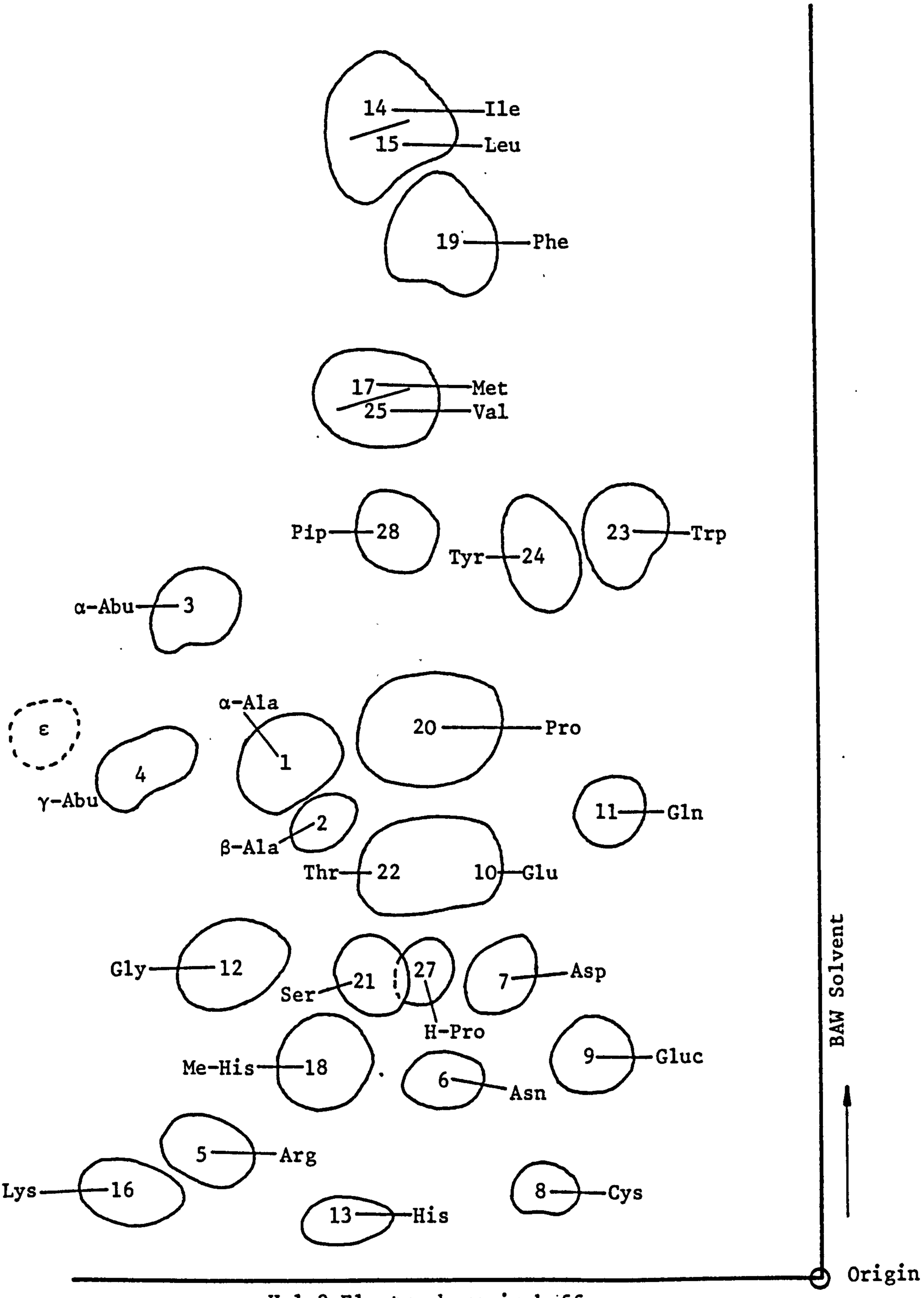


FIGURE 12

Amin acid map of U.K. survey No. 164 - *Trifolium repens*.

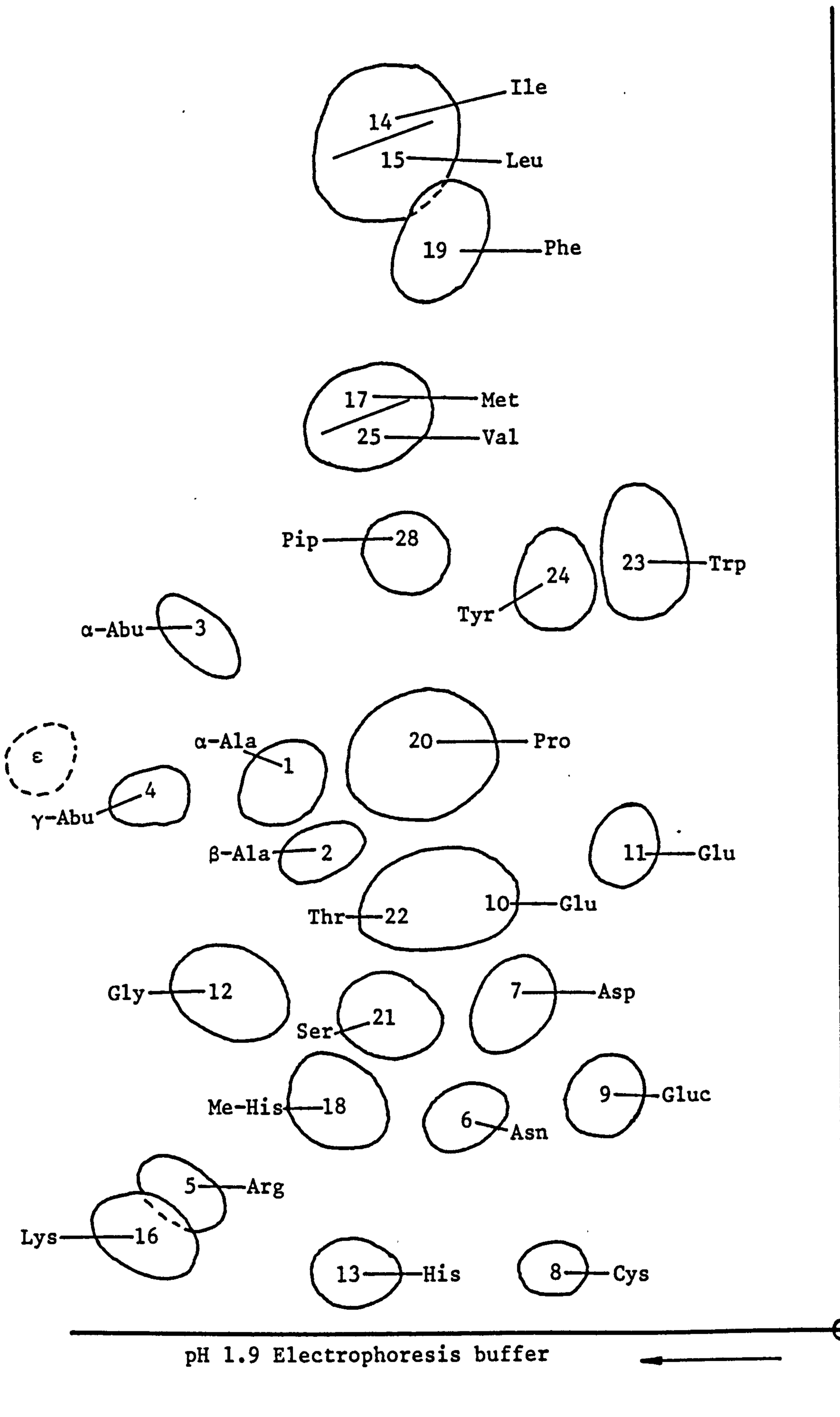


TABLE 37

Frequency of distribution of pollen grains in samples of *Castanea* honey (ex. U.K. survey)

Floral genus	Pollen (%)[2,3]								
	Honey sample code No[1]								
	91	39	89	178	36	97	29	84	43
Achillea									
Allium									
Brassica	0.2		0.8				2.2		
Calluna		0.2							
Carduus			0.2						0.1
Castanea	94.6	86.7	85.3	81.9	81.4	79.4	75.4	74.3	72.3
Corylus									
Epilobium							0.5		
Heracleum	0.7						0.3	0.5	0.4
Ligustrum									
Lotus						7.9			
Malva									
Prunus/pyrus									
Rubus	0.4		0.5				0.9	3.3	2.5
Sambacus									
Tilia	0.2	1.5	1.8						
Trifolium repens				6.7		0.5		6.0	7.0
Vicia					0.5				
Shrivelled pollen	3.4	9.6	10.8	8.9	15.8	12.2	18.2	15.1	16.1
Unidentified pollen	0.4	2.0	0.5	2.5	2.0	0.0	2.4	0.7	1.5
Total pollen grains counted	445	871	389	282	183	189	578	397	838
RQQNo4[4]	SC	B,SC, Hr	L,B	S,L,C, B	Wh, Bl	GF, Hr	B	NS	Bm,C, E

Cont'd....

For notes to key 1, 2, 3 and 4 refer to Table 31.

Table 37 cont'd.....

Floral genus	Pollen (%)[2,3] Honey sample code No[1]							
	83	28	90	2	25	107	59	23
Achillea					1.2			
Allium							5.8	
Brassica		13.0	3.2				0.6	
Calluna								
Carduus							0.6	
Castanea	68.2	63.7	61.5	60.4	59.4	57.9	57.6	53.2
Corylus							0.4	
Epilobium								
Heracleum	0.2	0.8			1.3			
Ligustrum				2.0				1.7
Lotus				6.0	-			
Malva				0.3				
Prunus/pyrus							5.4	4.7
Rubus	2.5	1.2	14.6	0.8	0.9	1.4	1.3	3.0
Sambacus				3.0				
Tilia			4.0	8.0				2.0
Trifolium repens	12.4			1.5	20.2		9.9	5.0
Vicia					1.7		1.3	
Shrivelled pollen	16.2	19.2	11.5	8.5	14.0	31.0	14.6	23.5
Unidentified pollen	0.4	1.6	5.2	8.5	1.2	9.6	2.5	6.7
Total pollen grains counted	444	511	348	270	762	145	465	718
RQQNo4[4]	B,L	B	Bm,L	L	Sc,L, B	B,Wh	H	L

TABLE 38

Free amino acids in *Castanea* honey

Amino Acids	Concentration (nMoles per g of honey)																
	CNo[1] → 91	39	89	178	36	97	29	84	43	83	28	90	2	25	59	107	23
Lys	122	T	163	111	152	230	182	176	T	97	251	75	81	147	116	82	95
Asp	129	373	204	174	66	190	285	205	174	192	209	52	92	177	106	116	148
Thr	43	59	97	40	58	103	42	135	288	138	233	25	82	166	124	67	170
Ser	124	114	107	81	50	168	131	127	184	103	162	38	46	149	118	114	93
Glu	432	375	393	92	73	467	528	245	375	242	450	65	170	421	253	236	256
Pro	4610	2390	2560	4470	2360	5010	2875	2140	3910	2160	2800	2470	1660	3980	4230	4470	3110
Gly	112	453	57	90	39	81	95	75	109	73	101	23	96	83	95	55	44
Ala	197	166	111	118	70	183	134	156	163	136	143	74	76	160	157	108	97
Val	52	32	75	53	30	110	60	56	93	51	85	24	21	76	111	112	71
Ile	37	49	71	98	22	80	55	33	93	44	70	22	26	141	114	71	34
Leu	40	36	35	72	166	57	44	26	95	312	95	24	22	97	120	68	36
Tyr	95	309	65	55	64	206	65	27	123	49	136	93	119	101	92	56	48
Phe	171	175	279	217	85	418	129	212	123	192	148	99	58	222	259	154	99
Sample[2] Date	9/82	9/81	8/81	8/82	8/81	8/82	8/81	8/81	8/81	8/81	8/81	8/80	7/81	8/81	8/82	8/82	9/81
Heating[3]	No	No	No	Yes	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No
Sugar[4] Feeding	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes

For notes to key 1, 2, 3, 4 and T, refer to footnote to Table 32.

TABLE 39

Occurrence of ninhydrin positive substances in *Castanea* Honeys

Ninhydrin positive substance	Sample numbers in which given compound was found not* to occur
β-Ala	28, 59, 83, 84
γ-Abu	28
Arg	2, 36, 39, 83, 84, 90, 178
Asu	36, 89, 90, 91, 96
Gluc	36, 43, 178
Gln	23, 91
Me-His	59, 84
Trp	28

In addition to the other amino acids found in all samples, the following ninhydrin positive substances were also present. These were: hydroxyproline in sample numbers 25, 28, 29, 36, 39, 43, 59, 83, 84, 89, 91 and 97; the unidentified spot 'x' in sample numbers 2, 23, 25, 29 and 178 and the unidentified spot 'p' in sample numbers 39 and 42. One other unidentified spot 'ε' was present in sample number 107.

* = Details as per Table 33.

FIGURE 13

Amino acid map of U.K. survey No. 25 - *Castanea*.

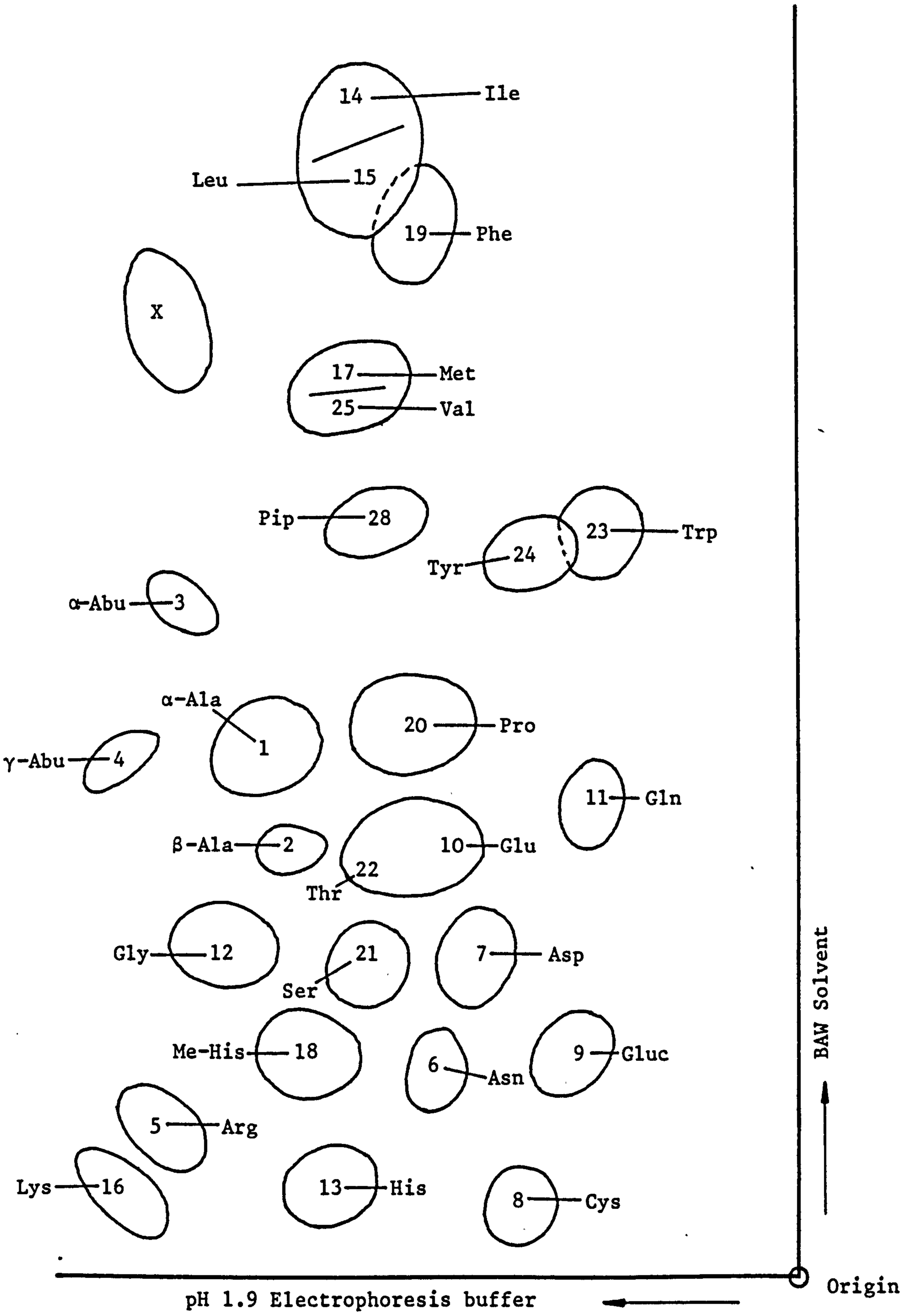


TABLE 14

Amino acid map of U.K. survey No. 107 - *Castanea*.

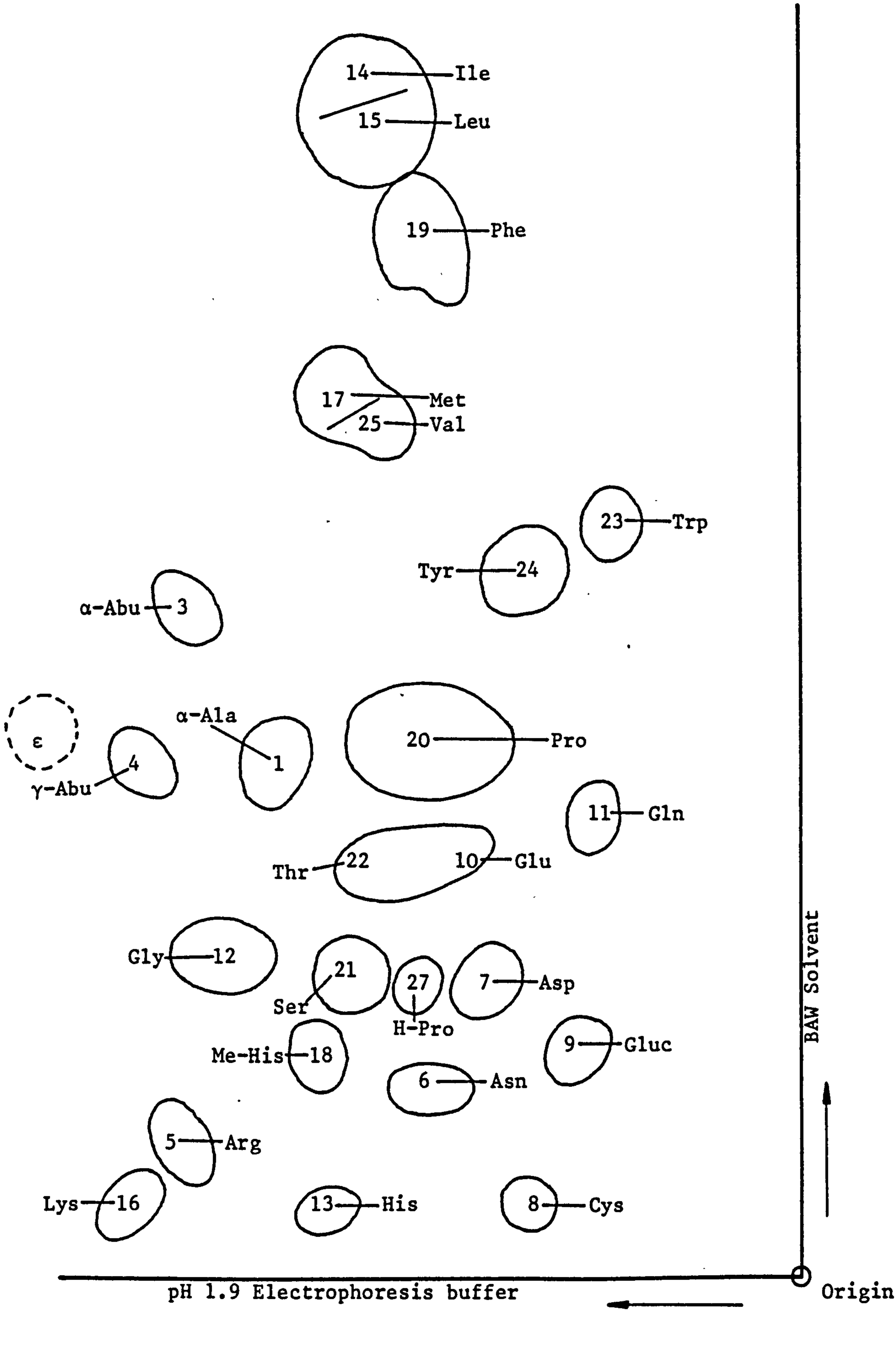


TABLE 40

Frequency of distribution of pollen grains in samples of *Calluna* honey (ex. U.K. survey)

Pollen (%)[2,3]	Honey sample Code No[1]							
	152	41	31	147	57	187	32	3
Brassica		0.4		3.0			23.9	
Calluna	91.0	86.1	84.3	80.6	74.7	58.7	57.0	52.5
Carduus						1.4		
Castanea				0.7	1.3			13.0
Compostae		0.9						
Hypericum								0.7
Ligustrum								0.7
Lotus								23.2
Phlox				0.7				
Taraxacum		2.2						
Trifolium repens	0.4	4.7			11.2	21.7		
Urtica								3.0
Vicia	0.4							
Shrivelled pollen	6.6	5.1	15.0	13.0	12.2	9.4	16.5	4.5
Unidentified pollen	1.6	0.5	0.4	2.0	0.2	8.7	2.5	2.0
Total pollen grains counted	225	800	223	268	376	286	242	284
RQQNo4[4]	NS	Lg,Hr	NS	Hr	Hr	NS	CV	Lg

For notes to key 1, 2, 3 and 4, refer to Table 31.

TABLE 41

Free amino acids in *Calluna* honey

Amino Acids	Concentration (nMoles per g of honey)							
CNo[1]	152	41	31	147	57	187	32	3
Lys	13	143	95	17	102	T	120	133
Asp	77	125	63	71	380	59	112	101
Thr	25	51	32	30	50	19	39	93
Ser	49	65	69	57	60	45	51	98
Glu	99	121	110	124	88	120	176	170
Pro	1350	1470	1310	1540	3240	2350	1470	2220
Gly	26	58	71	32	45	51	32	49
Ala	28	81	64	53	85	82	65	100
Val	T	43	35	T	50	32	43	61
Ile	T	50	26	T	31	48	31	47
Leu	T	41	89	T	35	33	24	44
Tyr	77	156	47	111	104	T	307	146
Phe	T	117	191	T	115	142	180	124
Sample[2]								
Date	Un	9/81	Un	Un	9/81	Un	12/81	9/81
Heating[3]	Un	No	Un	Un	No	Un	No	No
Sugar[4] Feeding	Un	Yes	Un	Un	Yes	Un	No	No

Notes to key 1, 2, 3, 4, T and Un - refer to footnote to Table 32.

TABLE 42

Occurrence of ninhydrin positive substances in *Calluna* honeys

Ninhydrin positive substance	Sample numbers in which given compound was found not* to occur
β-Ala	3, 32, 57
α-Abu γ-Abu	152
Arg	57
Asn	31, 32, 57
Gluc	147, 152
Gln	152
'x'	3, 32, 57

In addition to the other amino acids found in all samples, the following ninhydrin positive substances were also present. These were: pipecolic acid in sample number 32 and the unidentified spot 'p' in the sample numbers 3 and 31.

* = Details as per Table 33.

FIGURE 15

Amino acid map of U.K. survey No. 41 - *Calluna*.

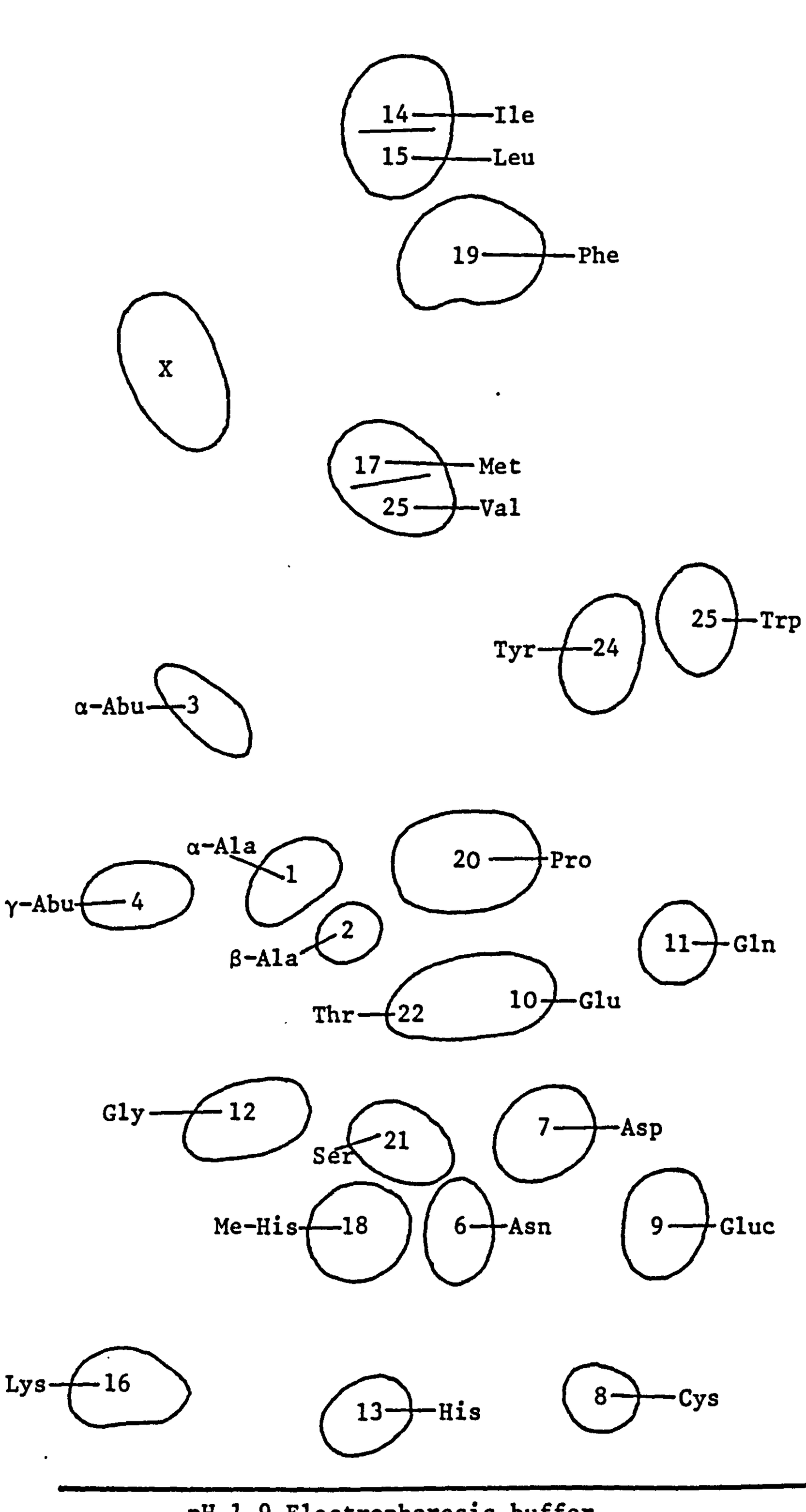


FIGURE 16

Amino acid map of U.K. survey No. 187 - *Calluna*.

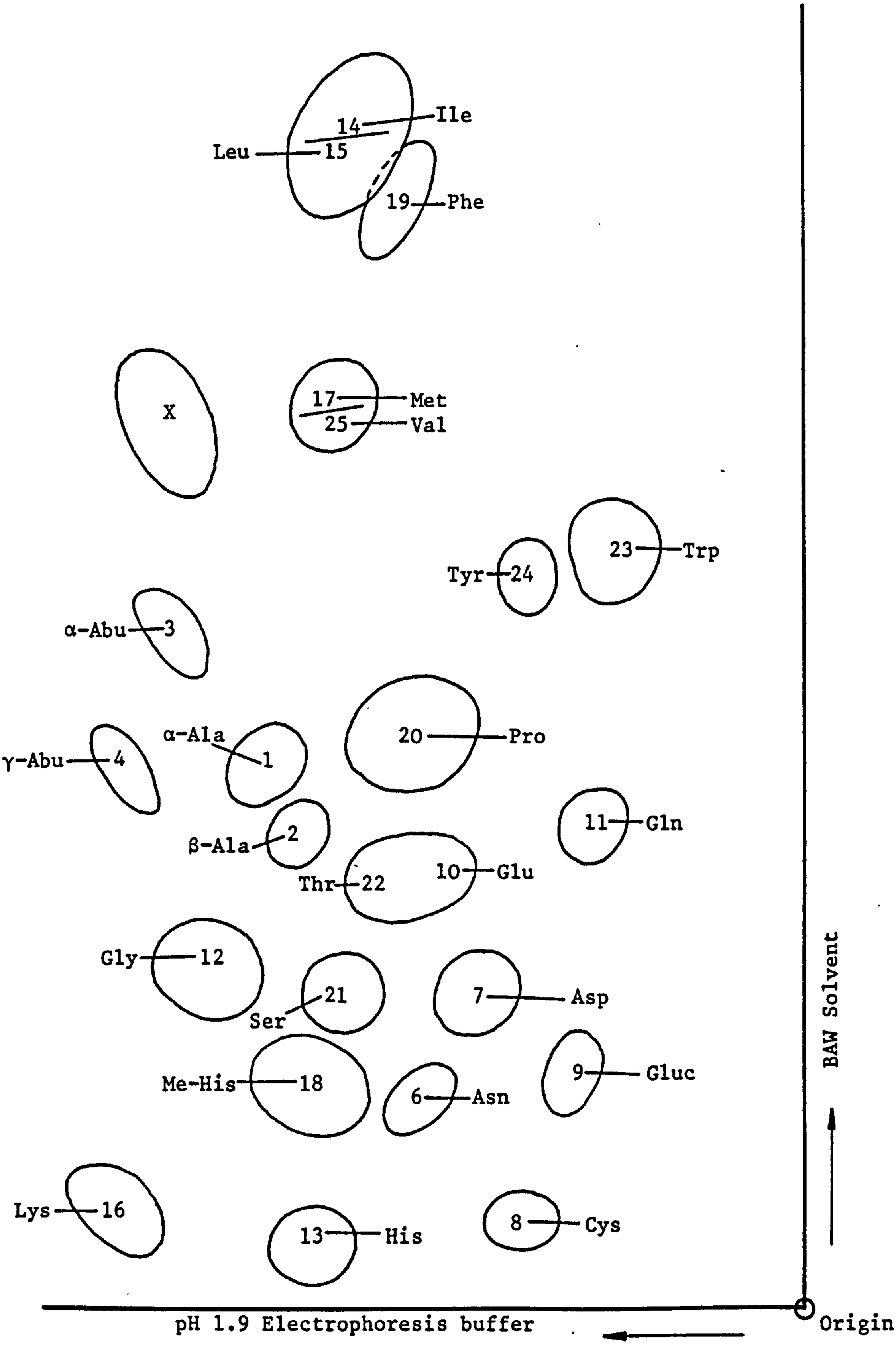


TABLE 43

Frequency of distribution of pollen grains in samples of *Myosotis* honey (ex. U.K. survey)

Floral genus	Pollen (%)[2,3] Honey sample code No[1]							
	106	120	121	125	129	170	171	177
Brassica	1.0			15.0	28.0	5.7	5.7	
Castanea				0.4	3.1	17.4	p	
Cotoneaster							8.9	
Helleborus				1.8				
Ilex			0.8					
Myosotis	95.0*	95.0*	73.0	63.7	95.0*	95.0*	95.0*	95.0*
Prunus/pyrus	1.0	8.0		3.2	37.3	30.4	5.7	p
Rubus		6.8				11.4	49.6	p
Salix					3.5			
Trifolium repens		1.8	11.1					
Shrivelled pollen	1.0	21.6	13.5	8.2	17.8	25.4	19.1	
Unidentified pollen	1.0	6.2	1.6	7.7	10.2	9.7	11.0	
Total pollen grains counted	VE	72	126	220	225	299	282	VE
RQQNo4[4]	S,FB	OR,C, B,Wh	FB,C HC,S	LG,F SF	GF, L	B,Pv	F,L, Wh,GR	Bs,S, H,A, PrCh

For notes to key 1, 2, 3 and 4 - refer to Table 31.

* - The amount present were visually estimated as seen through the microscope eyepiece.

VE = visually estimated.

p = pollen grain present but not counted.

TABLE 44

Free amino acids in *Myosotis* Honey

Amino Acids	Concentration (nMoles per g of Honey)							
CNo[1] →	106	120	121	125	129	170	171	177
Lys	83	54	161	132	86	124	106	113
Asp	140	150	173	115	107	176	161	148
Thr	77	24	91	40	68	46	50	75
Ser	71	76	141	96	79	85	68	100
Glu	208	204	340	210	156	228	181	221
Pro	3170	2720	6450	2420	3380	2790	1760	3110
Gly	41	56	94	52	108	65	44	64
Ala	101	83	164	131	127	120	87	99
Val	80	77	126	82	76	75	63	74
Ile	54	33	164	71	72	58	45	72
Leu	51	43	99	49	72	54	43	54
Tyr	59	23	64	81	113	58	79	126
Phe	384	90	188	415	147	194	113	209
Sample[2] date	5/82	8/82	8/82	6/82	7/82	8/82	8/82	7/82
Heating[3]	No	No	Yes	No	No	No	No	No
Sugar[4] feeding	Yes	Yes	Yes	No	Yes	Yes	Yes	No

Notes to key 1, 2, 3 and 4 refer to footnote to Table 32.0

considered necessary to list those samples which did not contain a particular amino acid within a pollen group as those present would result in excessively long lists. These differences in occurrence of ninhydrin positive substances have been listed in the order of Tables 32, 36, 39, 42 and 45. Also due to the sensitivity of the paper electrophoresis and chromatography combination basic amino acids such as arginine, histidine and others such as methionine were more evident on chromatograms even at low levels of concentration not detected by the automatic amino acid analyser. Typical examples of two amino acid chromatograms for each of the pollen groups have been shown in Figures 9 to 18.

The remaining four pollen groups of the nine major floral sources, that is, *Aesculus*, *Impatiens*, *Vicia* and *Clematis* accounted for the remaining predominant pollen of only seven samples. The format of arrangement of these honey samples was the same as described previously. The pollen content and the responses to questionnaire question 4 have been listed in Table 46. The amino acid concentration measurements and responses to questionnaire questions 3, 5 and 6 have also been given in Table 47. The following ninhydrin positive substances were detected on the amino acid maps. These were: α- and β-alanine, α- and γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, glucosamine, glutamine, glutamic acid, glycine, histidine, hydroxypipecolic acid, hydroxyproline, isoleucine, leucine, lysine, methionine, methylhistidine, phenylalanine, pipecolic acid, proline, serine, threonine, tryptophan, tyrosine, valine and unidentified spot 'x'. The differences in occurrence of ninhydrin positive susbtances have been given in Table 48.

TABLE 45

Occurrence of ninhydrin positive substances in *Myosotis* honeys

Ninhydrin positive substances	Sample numbers in which given compound was found not* to occur
Arg	120, 171
Cys	120
Gluc	170, 171
Gln	120, 170
'x'	106, 120, 121

In addition to the other amino acids found in all samples, the ninhydrin positive substances hydroxyproline and unidentified spot 'ε' were present in sample number 121.

* Details as per Table 33.

FIGURE 17

Amino acid map of U.K. survey No. 106 - *Myosotis*.

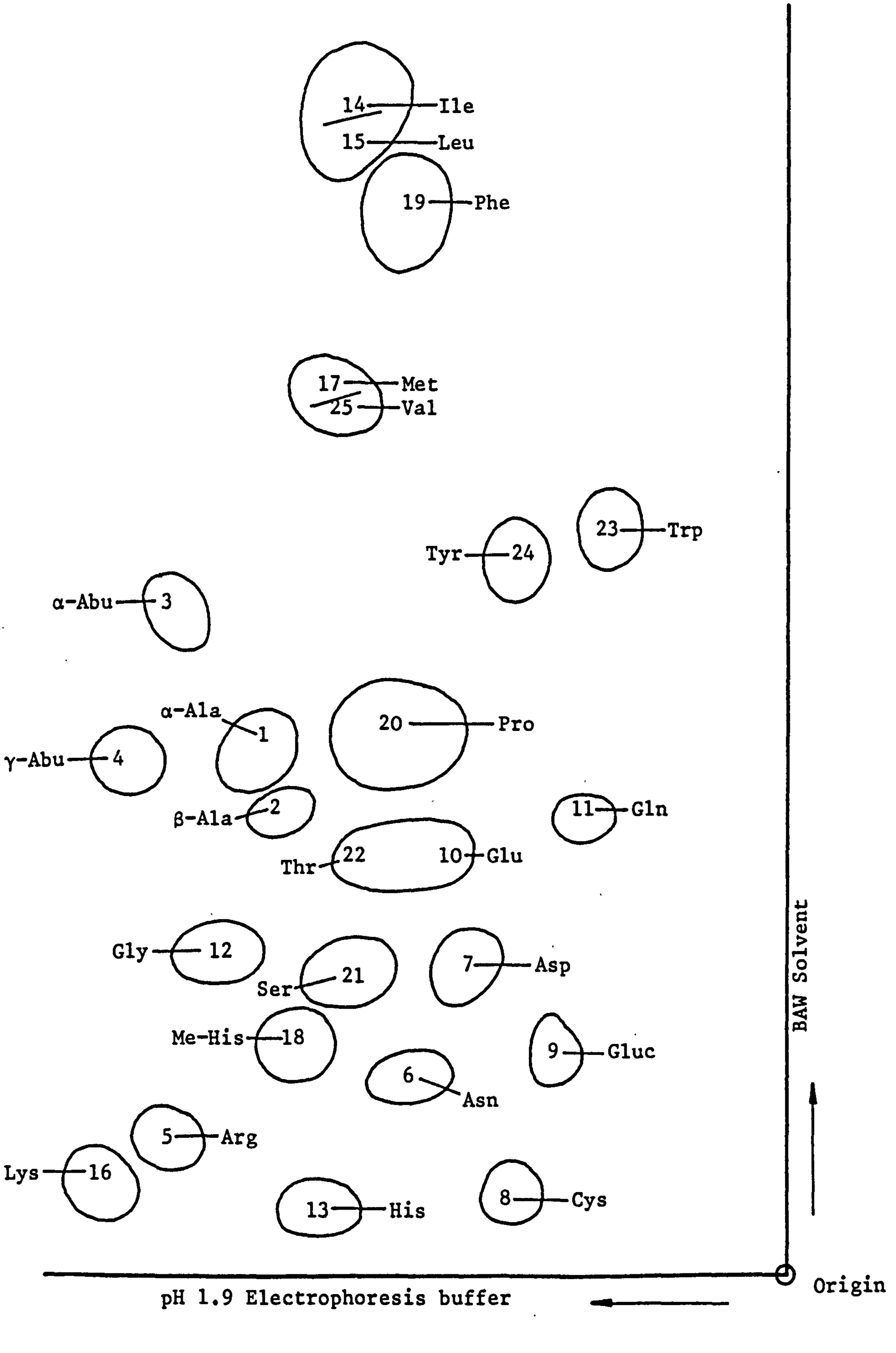


FIGURE 18

Amino acid map of U.K. survey No. 121 - *Myosotis*.

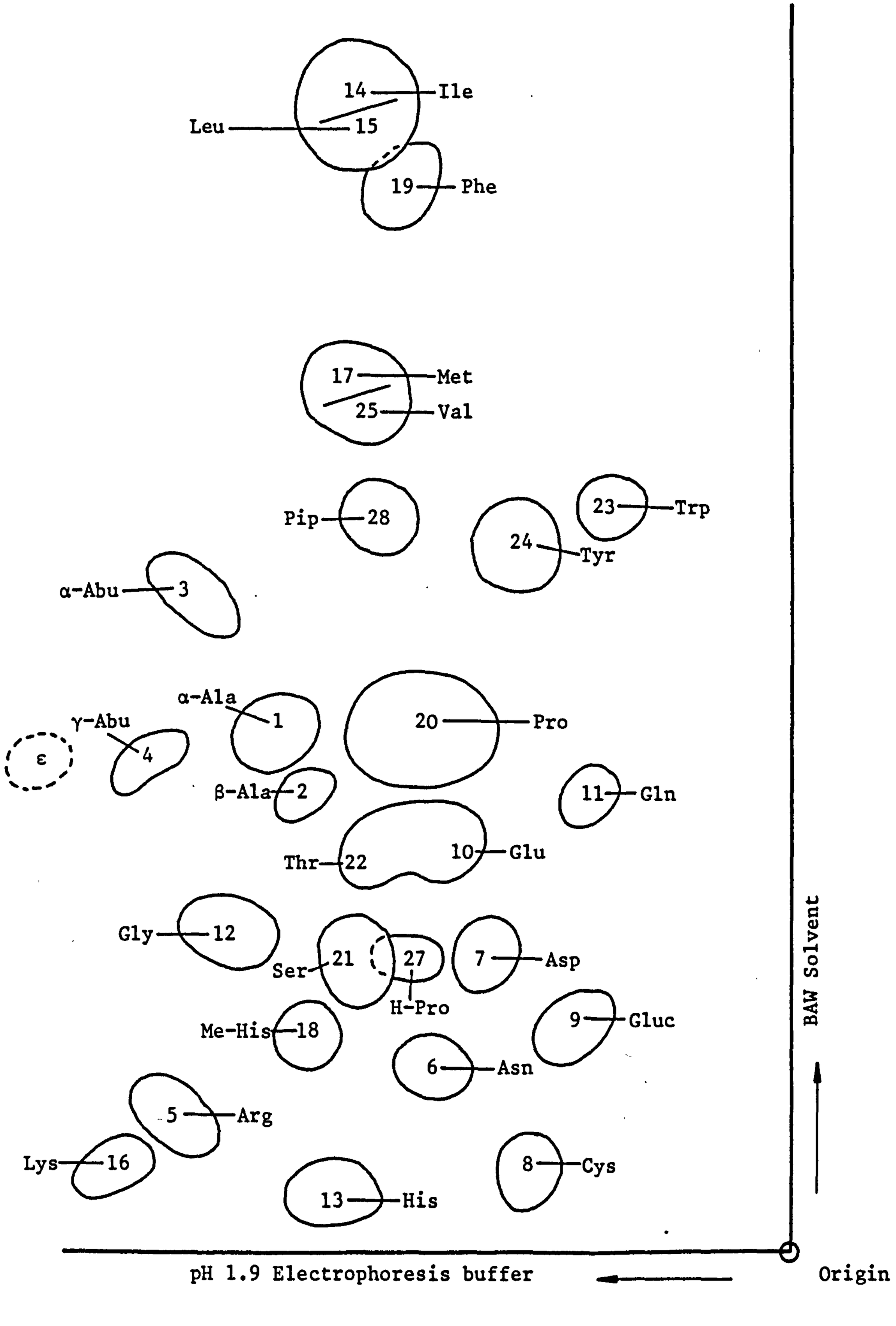


TABLE 46

Frequency of distribution of pollen grains in samples of predominant pollen honey (ex. U.K. survey)

Floral genus	Pollen (%)[2,3] Honey sample code No[1]						
	13	1	156	105	159	12	70
Achillea		1.0					
Aesculus	87.0	55.3					
Brassica				2.2		32.0	3.0
Clematis							58.4
Cotoneaster			13.8	12.0			
Heracleum	0.2	2.0				1.0	
Impatiens					54.9		
Ligustrum					4.3		
Lotus		8.0					
Prunus/pyrus	0.6		1.3			1.5	
Quercus		0.3					
Rubus			68.8	60.7			19.8
Tilia		0.7					0.8
Trifolium repens		0.7	3.8	1.4	23.2		4.3
Urtica		19.0					
Vicia	1.0					56.9	
Shrivelled pollen	9.0	6.5	9.0	18.9	13.7	5.6	9.7
Unidentified pollen	2.2	6.5	3.2	4.7	3.9	3.0	3.7
Total pollen grains counted	809	273	311	275	233	418	349
RQQNo4[4]	Rw,Wh	LBS, SF	GB, Rbw	GF	Rbw, WB	Bn	L,Bm,C Hz,Ch,W

For notes to key 1, 2, 3 and 4 - refer to Table 31.

TABLE 47

Free amino acids in predominant pollen type honeys

Amino Acids	Concentration (nMoles per g of Honey)						
CNo[1] →	13	1	156	105	159	12	70
Lys	208	216	122	87	140	156	80
Asp	247	173	128	328	48	43	169
Thr	530	388	61	46	70	116	138
Ser	324	173	131	103	96	48	115
Glu	545	397	212	250	151	395	281
Pro	3190	2970	3010	4280	4570	2110	2780
Gly	95	76	83	64	64	29	95
Ala	131	164	169	167	115	39	146
Val	80	63	79	86	60	30	66
Ile	56	51	84	43	41	19	59
Leu	65	57	104	38	59	14	43
Tyr	93	99	80	48	33	31	51
Phe	76	182	146	214	77	45	176
Sample[2] date	8/81	7/81	7/82	5/82	8/82	6/81	4/82
Heating[3]	No	No	No	No	No	No	No
Sugar[4] feeding	Yes	No	Yes	Yes	Yes	Yes	No

Notes to key 1, 2, 3 and 4, refer to footnote to Table 32.

TABLE 48

Occurrence of ninyydrin positive substances in the predominant pollen type honeys

Ninhydrin positive substances	Sample numbers in which given compounds were found not* to occur
β-Ala	13, 156
Cys	105
Gluc	159
Gln	1, 159
Me-His	1, 12, 13, 70
Trp	70, 159
'x'	1, 70, 105

In addition to the other amino acids found in all samples, the following ninhydrin positive substances were also present. These were: hydroxyproline in sample numbers 70 and 156; hydroxypipecolic acid in sample number 70; pipecolic acid in sample numbers 1 and 70 and the unidentified spot 'p' in sample number 105.

* Details as per Table 33.

Secondary pollen type

Honey samples containing the secondary frequency class pollen, that is, those containing between 16% and 45% pollen were allocated in this category. There were twenty one samples that accounted for seven secondary floral pollens. These seven pollens were: *Brassica*, *Aesculus*, *Trifolium repens*, *Rubus*, *Castanea*, *Teucrium* and *Alnus*. The format of arrangement of these honey samples was the same as described before. The pollen contents and the response to questionnaire question 4 have been listed in the order as given in Table 49. The amino acid concentration measurements and the responses to questionnaire questions 3, 5 and 6 have been listed in the order as given in Table 50.

The ninhydrin positive substances detected on the amino acid maps were: α- and β-alanine, α- and γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, glucosamine, glutamine, glutamic acid, glycine, hydroxyproline, histidine, isoleucine, leucine, lysine, methionine, methyl-histidine, phenylalanine, pipecolic acid, proline, serine, threonine, tryptophan tyrosine, valine and the two unidentified spots 'x' and 'p'. The differences in occurrence of ninhydrin positive substances were evaluated within and between each pollen group and these have been listed in Table 51. Further, there were two other unidentified ninhydrin positive substances detected in sample code number 169 and these were labelled as 'θ' and 'Ω'. Their position of occurrence relative to the other spots have been shown in Figure 19.

Secondary multiple pollen type

Honey samples which contained two or more secondary frequency class pollens were allocated to this category. There were forty nine honey samples which accounted for twelve pollen groups listed in the order

TABLE 49

Frequency of distribution of pollen grains in samples of secondary pollen type honeys (ex. U.K. survey

Floral Genus	Pollen (%)[2,3] Honey sample code no[1]										
	76	69	176	24	30	62	79	133	127	99	80
Achillea			9.9	1.9							
Aesculus										10.1	
Brassica	44.8	36.5	4.4	1.9	7.3		4.4	2.3	1.3		12.6
Calluna						6.2					
Castanea			40.5	38.0	28.0			9.0		6.6	8.6
Cotoneaster											
Heracleum		3.0	3.6	4.0					1.8	0.3	
Ligustrum				1.4	3.8						
Melilotus					15.0				13.7		
Prunus/pyrus	12.7	11.6		5.0		13.4		0.8	11.5	4.5	
Rubus	6.6	3.2	5.2	1.4	8.6		9.5				5.3
Taraxacum					7.6	4.1					
Tilia	0.8				2.8		1.5				
Trifolium pratense						8.2					
Trifolium repens	10.7	15.4		2.8		44.3	44.1	42.0	38.5	37.9	34.4
Vicia		12.0									
Shrivelled pollen	18.5	14.0	27.9	37.0	23.2	18.5	33.8	35.7	23.9	35.5	30.5
Unidentified pollen	5.8	4.0	8.5	6.6	3.8	5.1	6.6	9.4	9.3	5.1	8.6
Total pollen grains counted	243	370	365	213	289	97	136	255	226	335	151
RQQNo4[4]	G,F, Ce	NS	NS	L	NS	NS	Wh,B	OR	NS	P,GF	B,Ce

For notes to key 1, 2, 3 and 4 - refer to Table 31.

TABLE 49 Cont'd.

Floral Genus	Pollen (%)[2,3] Honey sample code no[1]									
	149	14	19	17	148	15	104	5	55	169
Achillea								2.0		13.9
Aesculus							33.2			
Alnus										38.0
Brassica	9.5			6.0	14.0		0.9		10.9	
Calluna				0.7						
Castanea	5.6		13.0		9.1					
Carduus			0.8	0.7						
Chenopodium										3.7
Endymion							6.2			
Epilobium			3.0					0.5		
Heracleum		0.8				2.0				
Hypericum		3.0		3.0		5.5		11.0		
Ilex								6.0		
Impatiens										1.5
Ligustrum			11.0						0.8	
Lotus		5.0				8.0				
Malva								0.5		
Myrtaceae**										12.6
Prunus/pyrus							11.8	1.5	3.4	
Rubus	4.7	11.0	6.5	6.5		12.0		27.7		
Taraxacum								1.5		
Teucrium									33.6	
Tilia			5.0							
Trifolium repens	30.2	26.8	23.9	22.1	21.6	20.9			10.1	
Urtica	4.7	5.0				3.6				
Verbascum								4.0		
Vicia					14.0			5.5		
Shrivelled pollen	38.2	46.4	32.2	5.62	36.2	45.0	31.3	23.7	34.4	15.1
Unidentified pollen	7.1	2.0	4.5	4.0	5.0	3.0	16.6	16.0	6.7	15.1
Total pollen grains counted	338	610	472	290	342	522	211	202	119	324
RQQNo4[4]	C	B,Fw	L	FT,B C,BH	B	B,Fw	Bs	B,C, SL	Ws,Bm Bu	Sn

** - Identified at plant family level only.

TABLE 50

Free amino acids in honey with secondary pollen

Amino acids	Concentration (nMoles per g of honey)										
CNo[1] →	76	69	176	24	30	62	79	133	127	99	80
Lys	170	372	126	72	88	128	143	T	144	46	153
Asp	94	77	141	131	193	141	143	311	114	131	211
Thr	64	44	84	151	190	68	84	77	43	44	134
Ser	123	77	95	115	99	118	123	148	127	94	191
Glu	230	240	220	191	304	243	226	570	194	170	420
Pro	3550	2880	1440	1040	2570	3820	3610	3710	4970	3940	7710
Gly	59	45	76	218	61	97	60	181	90	74	85
Ala	131	84	122	145	111	182	120	158	174	192	207
Val	98	63	69	72	40	99	79	88	84	68	118
Ile	87	120	51	73	49	82	88	101	58	59	99
Leu	37	33	42	71	32	83	45	55	56	52	46
Tyr	68	130	70	240	44	104	74	94	126	79	61
Phe	245	216	254	99	114	289	216	131	394	226	176
Sample[2] date	6/81	8/82	8/82	9/81	Un	6/82	8/81	6/82	8/81	9/82	8/81
Heating[3]	No	No	No	Yes	Un	Yes	No	Yes	No	No	No
Sugar[4] feeding	Yes	Yes	Yes	Yes	Un	Yes	No	Yes	Yes	Yes	No

cont'd....

TABLE 50 cont'd...

Amino acids	Concentration (nMoles per g of Honey)									
CNo[1] →	149	14	19	17	148	15	104	5	55	169
Lys	186	223	104	138	245	155	53	424	92	151
Asp	298	70	99	58	379	88	148	72	35	59
Thr	110	109	118	112	86	90	41	205	17	T
Ser	323	62	99	72	288	61	80	148	28	T
Glu	546	127	160	387	710	145	175	201	83	19
Pro	6970	2610	2710	2520	3790	2910	3010	5200	2420	1060
Gly	116	54	38	51	136	50	61	82	34	56
Ala	214	105	76	112	246	113	141	152	35	91
Val	117	35	60	55	90	48	77	89	25	37
Ile	96	51	416	47	186	64	56	52	20	25
Leu	107	43	327	39	136	52	44	45	16	20
Tyr	93	39	51	2450	80	41	40	95	T	T
Phe	225	113	118	2450	242	100	88	450	89	66
Sample[2] date	Un	8/81	8/81	10/81	Un	8/81	5/82	8/81	8/82	Un
Heating[3]	Un	Yes	No	Yes	Un	Yes	No	No	No	Un
Sugar[4] feeding	Un	Yes	No	No	Un	Yes	Yes	Yes	Yes	Un

Notes to key 1, 2, 3, 4, T and Un, refer to footnotes to Table 32.

TABLE 51

Occurrence of ninhydrin positive substances in secondary pollen type honeys

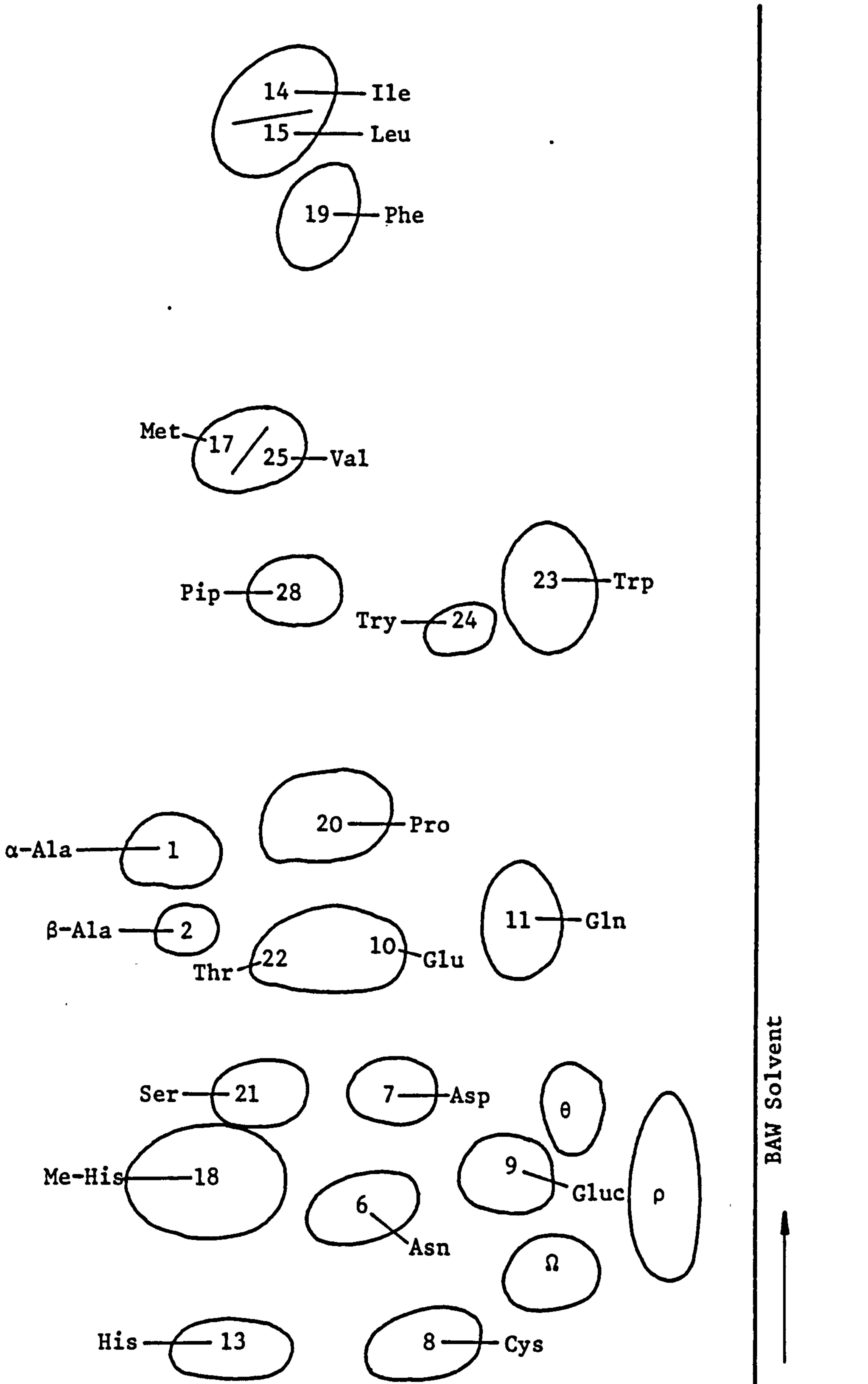
Ninhydrin positive substances	Sample numbers in which given compound was found not* to occur
β-Ala	24, 79, 80
Arg	17, 55, 69, 76, 99, 104, 127, 169
Asn	55, 69, 76, 104
Cys	55
Gluc	14, 15, 104
Glu	5, 14, 15, 19, 176
Me-His	79, 80
Trp	112b
'x'	30, 55, 169
'p'	76, 176

In addition to the other amino acids found in all samples, the following ninhydrin substances were also present. These were: hydroxyproline in sample numbers 17, 79, 99 and 148; pipecolic acid in sample numbers 62, 80 and 99; unidentified spot 'x' in sample numbers 149 and 176; unidentified spot 'p' in sample number 56 and two other unidentified spots 'θ' and 'Ω' in sample number 169.

* Details as per Table 33.

FIGURE 19

Amino acid map of U.K. sample No. 169



as given in Table 52. From the two pollens identified in each sample, the pollen with the highest percentage value was used for arranging the samples in the format described previously. The pollen contents and responses to questionnaire question 4 have been listed in Table 52. The amino acid concentration measurements and the responses to questionnaire questions 3, 5 and 6 have been given in Table 53 in the order shown in Table 52.

The ninhydrin positive substances detected on the amino acid maps were: α- and β-alanine, α- and γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, glucosamine, glutamine, glutamic acid, glycine, hydroxypipecolic acid, hydroxyproline, histidine, isoleucine, leucine, lysine, methionine, methyl-histidine, phenylalanine, pipecolic acid, proline, serine, threonine, tryptophan, tyrosine, valine and the two unidentified spots 'x' and 'p'. The differences in occurrence of ninhydrin positive substances were evaluated within each pollen group and these have been listed in Table 54. There were two honey samples which did not belong to the secondary multiple pollen type category, because these two samples contained important minor frequency class pollen, that is, those samples containing between 3% and 15% pollen. It was considered appropriate to insert these two samples Code Nos. 16 and 33 at the end of the secondary multiple pollen group list rather than base a section on two samples which were inadequate in number for floral categorisation.

Unidentified pollen type

There were eight honey samples which did not contain enough honey for pollen extraction and subsequent identification and these were allocated to this category. The amino acid concentration measurements

TABLE 52

Frequency of distribution of pollen grains in samples of secondary multiple pollen type honeys (ex. U.K. survey)

Floral Genus	Pollen (%)[2,3] Honey sample code no[1]																	
	71	9	146	173	139	22	46	75	10	52	53	168	94	96	122	112	21
Achillea						1.5	0.6		0.5								
Aesculus	31.5																
Brassica	16.6	43.2	39.4	34.4	28.9	16.8		8.0		5.2	3.2		19.4				1.0
Calluna	1.6	0.1					43.9	42.7	35.7	32.1	35.9						
Cardicus		0.1															
Castanea				9.4		15.3					9.0	36.1	35.3	32.7	32.5	26.5	19.5
Epilobium		0.5						0.8									
Fagopyrum					18.4												
Fragaria														18.2			
Heracleum		2.0	0.7								0.5						
Hypericum						0.8			6.0								
Ilex		0.3															
Ligustrum							1.2										1.0
Myrtaceae**	3.3																
Prunus/pyrus	8.7	10.0	2.0	2.9	1.3	2.6							3.2	16.7	19.0		18.5
Rubus	2.0					1.0								2.9	11.2	18.6	11.0
Taraxacum		0.3				1.5				0.2							2.0
Tilia						1.5						15.0					
Trifolium repens	17.8	26.4	32.3	30.3		5.0	35.1	25.5	31.8	18.8		17.3	4.6			18.6	8.0
Urtica					25.0						31.9						
Vicia			1.3		3.9												
Shrivelled pollen	13.7	12.0	14.8	16.2	18.4	47.5	18.0	17.6	18.0	43.0	16.6	29.5	35.7	26.3	26.9	35.0	26.0
Unidentified pollen	4.6	5.0	9.4	6.8	3.9	6.0	1.2	5.4	6.0	0.6	2.7	2.0	1.8	2.9	10.4	2.2	13.0
Total pollen grains counted	241	643	297	340	76	463	496	239	361	479	373	346	283	406	268	489	200
RQQ No4[4]	L,He	Ç,L	Tr,L	C,B	FB	NS	C,L, G	Hr	CV	CV	Hr	L,WL	NS	W,Mx, G,Ce	NS	BM	C,B Wh

** Identified at plant family level only

TABLE 52 Cont'd.

Floral Genus	Pollen (%)[2,3] Honey sample code no[2]															
	72	85	111	190	50	60	113	161	63	4	123	18	119	191	65	37
Acer								9.0								
Achillea	2.4									1.5						
Brassica			8.9		23.3		21.3	2.6				3.0	23.0	20.8	14.4	17.7
Calluna		22.2													20.8	21.4
Carduus															1.5	
Castanea						1.7	24.7				15.9			7.9		
Chrysanthemum												16.0				
Cotoneaster	24.7							5.3			16.8					
Heracleum			5.0		2.1					0.5				12.0		
Helloborus									17.9							
Hypericum										24.3						
Ilex										8.3						
Impatiens	31.9															
Ligustrum	25.2															
Lilium					4.5											
Linaria		25.5														
Melilotus							7.2									
Prunus/pyrus	22.7			41.5	38.8	34.2	33.8	25.2	24.8		15.6		13.2			
Quercus												5.5				
Rubus	3.2				10.1				7.7	35.6	18.2					
Salix	3.6			7.0	2.5											
Taraxacum						0.8		5.6	6.0							11.5
Tilia		0.7									0.3	19.6				
*Trifolium inc**						12.7										
Trifolium repens	14.3		23.2	24.6	6.0	22.8		21.0				15.7	40.8	33.3	40.1	35.1
Vicia	5.6		13.9		0.5					2.0						
Shrivelled pollen	21.0	15.8	17.8	18.1	14.2	18.3	6.4	21.8	35.9	27.7	28.9	31.0	19.9	18.7	21.3	13.7
Unidentified pollen	2.4	4.1	5.8	8.8	4.6	2.8	6.6	9.4	7.7	0.1	4.3	11.0	3.1	7.2	1.8	0.6
Total pollen grains counted	251	266	259	342	394	465	361	266	117	205	346	127	287	390	389	713
RQQNo4[4]	A,H	C,Hr	V,Mx	NS	FB	S	SpF, F	D	NS	GF	L,Pr B,H	L,B WB	Bn,DR Rb	NS	Hr	Lg

* inc = *incarnatum*

TABLE 52 Cont'd.

Floral Genus	Pollen (%)[2,3] Honey sample code no[1]															
	38	167	172	128	48	93	86	49	78	77	181	189	136	109	16	33
Achillea								3.7								
Brassica	1.5					16.7		3.7	3.5	10.8	40.6	24.7	26.3	18.4	0.7	7.6
Calluna						6.9	0.9									
Carduus									2.1	3.2						
Castanea	19.6	31.3	31.5					4.8							1.0	
Endynion							2.2									
Epilobium	0.3															
Heracleum				2.6					2.1					2.8		
Ilex															4.6	
Impateins	5.4															
Ligustrum	0.7				2.2											
Melilotus				4.3												
Prunus/pyrus		0.8		26.7	35.7	17.7	17.1	24.1		5.1	3.9	4.2	25.0	13.6		6.0
Rubus	1.6				4.4		7.6		18.6	17.2					5.0	9.5
Taraxacum			0.5					1.6								
Trifolium repens	42.1	37.7	36.0	37.9	37.5	34.4	34.2	29.9	34.9	34.4		12.6	5.2		14.0	6.0
Urtica														13.2		
Vicia						2.0					42.0	35.7	26.9	21.2		
Shrivelled pollen	26.5	24.9	27.5	18.9	16.9	13.8	31.5	31.5	35.2	25.8	18.6	12.9	10.7	26.4	66.0	65.0
Unidentified pollen	2.3	5.3	4.5	9.5	3.0	8.5	6.3	0.5	3.5	3.4	5.0	9.8	5.8	4.4	8.0	5.9
Total pollen grains counted	667	358	200	116	361	305	222	187	338	314	362	356	308	250	278	158
RQQ No4[4]	NS	F,D, Ch	C, B	NS	NS	Hr	C,WL	G,Ia	UF,BM not C	MxGF Sh	FB	C	FB	B,L, Wh	MxL,B WB	C,Bm

TABLE 53

Free amino acids in honey with secondary multiple pollen

Amino Acids	Concentration (nMoles per g of honey)																
	CNo[1] → 71	9	146	173	139	22	46	75	10	52	53	168	94	96	122	112	21
Lys	103	195	120	182	147	256	101	75	131	126	76	108	225	66	T	198	178
Asp	94	71	117	240	44	349	93	70	96	70	84	70	141	150	134	260	152
Thr	127	187	43	125	27	411	53	50	46	48	66	23	71	144	87	97	127
Ser	106	145	93	166	41	186	80	64	61	74	83	34	94	130	95	107	98
Glu	185	146	188	438	136	960	134	102	104	135	179	142	228	304	191	400	268
Pro	5230	4000	2110	2730	2780	2690	4400	1820	1460	1700	3810	2060	3130	4160	3050	4640	4460
Gly	65	60	88	83	22	125	68	31	43	30	48	42	49	50	32	80	60
Ala	167	120	113	196	50	200	151	89	65	80	151	48	123	195	75	156	112
Val	107	78	62	200	51	77	85	46	37	76	113	26	87	110	75	77	81
Ile	70	70	93	125	80	41	270	37	29	205	90	27	43	69	53	116	68
Leu	66	70	75	114	60	37	253	43	45	143	71	17	32	75	45	124	53
Tyr	79	56	63	224	43	185	75	43	90	57	37	T	59	200	31	44	80
Phe	369	173	245	580	95	154	175	240	78	176	550	120	132	214	122	38	17
Sample[2] Date	7/82	10/81	7/82	8/81	8/82	Un	7/82	10/81	8/81	10/81	9/82	8/81	8/82	5/82	5/82	8/81	8/82
Heating[3]	No	No	No	Yes	No	Un	No	No	No	No	Yes	No	No	No	No	Yes	No
Sugar[4] Feeding	No	Yes	Yes	Yea	Yes	Un	No	No	Yes	No	Yes	Yes	No	No	No	Yes	No

Notes to key 1, 2, 3, 4, T and Un refer to footnote to Table 32.

Table 53 cont'd....

Amino Acids	Concentration (nMoles per g of honey)															
	CNo[1] → 72	85	111	190	50	60	113	161	63	4	132	18	119	191	65	37
Lys	115	165	153	47	86	154	164	T	99	320	23	139	79	65	20	238
Asp	91	62	106	90	101	90	174	96	81	69	405	132	84	75	61	95
Thr	174	52	60	31	187	128	47	21	T	185	36	142	63	36	55	73
Ser	95	66	71	65	102	113	107	57	30	130	99	91	77	55	67	66
Glu	219	124	214	152	160	197	241	153	112	182	440	310	176	136	126	86
Pro	2240	3690	5010	2860	2140	4660	4290	2100	1990	3580	3640	2310	2870	3080	2150	1510
Gly	49	41	33	79	46	52	67	40	29	70	85	43	70	60	46	41
Ala	102	90	90	72	101	109	133	76	127	121	151	91	103	91	92	69
Val	85	41	86	58	75	72	95	62	44	85	70	52	70	53	43	29
Ile	54	36	50	46	46	62	75	34	45	46	35	63	90	213	51	36
Leu	52	44	54	54	41	58	81	34	49	39	32	52	49	110	35	39
Tyr	46	38	65	53	40	103	70	T	37	84	T	92	93	55	42	156
Phe	136	175	134	234	122	165	180	164	56	286	160	113	105	630	117	110
Sample[2] Date	6/82	9/81	8/82	Un	5/82	8/82	8/82	6/82	8/82	8/81	8/82	12/81	9/82	Un	9/81	9/81
Heating[3]	No	No	No	Un	No	No	Yes	No	No	No	No	No	Yes	Un	Yes	No
Sugar[4] Feeding	Yes	Yes	Yes	Un	No	No	Yes	Yes	No	Yes	No	No	Yes	Un	Yes	No

Cont'd....

Table 53 cont'd....

Amino Acids	Concentration (nMoles per g of honey)															
CNo[1] →	38	167	172	128	48	93	86	49	78	77	181	189	136	109	16	33
Lys	272	81	98	403	53	117	185	134	268	129	137	143	218	152	231	122
Asp	263	141	194	112	217	99	95	110	143	86	219	102	91	35	290	151
Thr	234	73	127	60	102	97	79	45	91	71	107	55	91	39	169	130
Ser	222	83	152	87	136	130	87	75	192	186	138	70	109	51	123	90
Glu	2110	348	368	231	381	215	148	206	279	213	324	182	235	128	510	140
Pro	4700	4730	4260	6680	5170	4140	1570	4060	5220	2960	3640	3140	2940	3110	3540	3840
Gly	107	73	85	86	110	45	70	69	65	57	113	59	74	56	73	66
Ala	217	126	206	162	173	136	113	122	145	123	114	94	112	97	142	137
Val	90	105	141	83	121	100	61	84	118	115	115	68	77	59	61	65
Ile	58	87	127	66	135	38	59	67	78	102	75	58	118	41	71	40
Leu	62	99	85	40	138	42	55	48	74	103	64	36	93	41	62	50
Tyr	62	160	269	135	67	157	43	70	124	73	83	62	107	95	252	107
Phe	250	303	580	486	148	112	123	196	218	211	155	120	300	117	271	153
Sample[2] Date	8/81	8/82	8/82	8/81	9/82	9/82	10/81	9/82	8/82	8/82	5/81	9/82	7/82	10/82	10/81	12/81
Heating[3]	Yes	No	No	No	Yes	No	No	Yes	No	No	No	No	No	No	Yes	No
Sugar[4] Feeding	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No

TABLE 54

Occurrence of ninhydrin positive substances in secondary multiple pollen honeys

Ninhydrin positive substances	Sample numbers in which given compound was found not* to occur
β-Ala	37, 38, 53, 65, 72, 75, 128, 190
Arg	16, 37, 48, 49, 52, 53, 63, 65, 93, 94, 109, 111, 119, 122, 123, 139, 161, 167, 168, 181, 189, 190
Asn	10, 21, 37, 49, 52, 65, 72, 85, 86, 93, 112, 161
Cys	9, 53, 60, 85, 93, 119, 139, 161
Gln	4, 72, 78, 94, 139, 167, 168, 173, 189
Gluc	48, 49, 72, 94, 122, 161, 167, 168, 172
Hyp	50, 113, 161, 118
Me-His	38, 50, 78
Pip	4, 16, 37, 50, 63, 72, 85, 93, 94, 128, 146, 161, 167, 168, 172, 190, 191
Trp	53, 72, 77, 167, 168, 173, 189
'x'	37, 38, 65, 71, 77, 78, 85, 123
'p'	119, 191

In addition to the other amino acids found in other samples, the following ninhydrin positive substances were also present. These were: hydroxypipecolic acid in sample number 71; hydroxyproline in sample numbers 71, 138 and 172; the unidentified spot 'x' in sample numbers 33, 46, 128, 161, 168 and 190 and the unidentified spot 'p' in sample numbers 9, 10, 16, 93, 113, 122 and 123.

* Details as per Table 33.

and responses to questionnaire questions 3, 5 and 6 have been listed in Table 55. The ninhydrin positive substances identified were: α- and β-alanine, α- and γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, glucosamine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, methyl-histidine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and the two unidentified spots 'x' and 'p'. The differences in occurrence of ninhydrin positive substances have been given in Table 56.

Moreover, sample Code No. 126 was not analysed as described because this honey was received in pieces of comb. There was not enough honey extracted from the combs for either amino acid or pollen analysis.

Amino Acids in the Foreign and Commercial Samples Arranged in Order of Stated Country Sources

The foreign and commercial honey samples were categorised according to their known country of origin, commercial processing and commercial sugar products rather than on the basis of their pollen content. The sixty four honey samples were allocated into three categories and these were known country; unknown country; suspect origin and English commercial and sugar products types.

Known Country Type

Honey samples in this category were grouped according to their stated country of origin. There were sixteen countries and between them they accounted for forty one samples of honey. These samples were arranged into sixteen country groups in an alphabetical order as listed in Table IB in Appendix I. The pollen contents of each honey sample belonging to each of these sixteen country groups have been given in Table 57. The amino acid concentration measurements and the information regarding

TABLE 55

Free amino acids in unidentified samples

Amino Acids	Concentration (nMoles per g of Honey)							
CNo[1] →	6	7	40	73	95	130	165	166
Lys	49	145	151	139	216	122	72	60
Asp	74	61	172	79	84	167	76	83
Thr	24	42	191	116	87	44	210	24
Ser	48	43	156	103	91	92	40	51
Glu	112	101	340	157	174	197	134	119
Pro	2240	1610	4370	3530	2740	4930	1640	2350
Gly	45	38	64	44	37	60	32	30
Ala	115	70	170	115	89	147	40	39
Val	41	34	102	91	85	71	T	T
Ile	32	29	58	71	40	60	12	13
Leu	43	36	68	78	30	60	14	14
Tyr	34	106	700	53	47	103	T	35
Phe	72	82	265	209	100	298	62	29
Sample[2] date		6/81	9/81	7/82	8/82	11/82	8/82	8/82
Heating[3]		No	No	No	No	No	No	No
Sugar[4] feeding		Yes	Yes	No	No	Yes	Yes	Yes

Notes to key 1, 2, 3, 4 and T, refer to footnote to Table 32.

TABLE 56

Occurrence of ninhydrin positive substances in unidentified honey samples

Ninhydrin positive substance	Sample numbers in which given compounds was found not* to occur
β-Ala	7, 40, 166
Arg	6, 40, 95, 165, 166
Asn	40, 73
Cys	7, 40
Gluc	95, 130
Trp	165
'x'	165, 166

In addition to other other amino acids found in all samples, the ninhydrin positive substance hydroxyproline was present in sample number 73.

* Details as per Table 33.

TABLE 57

Frequency of distribution of pollen grains in samples of known country type honeys (ex. foreign and commercial).

Floral Genus	Pollen (%)[2,3] Honey sample code No[1]													
	193	194	195	196	197	198	199	200	201	202	203	204	205	206
Acacia													0.3	
Banksia		21.9		0.3									0.6	55.3
Brassica		3.9		5.2		2.0	46.8	27.7	61.5	70.7			25.3	
Castanea	63.9													
Cirsium	2.9					1.1		3.2		4.1			1.7	
Echium		6.0	78.2	73.8	8.0			13.9						
Erica	1.9													
Eucalyptus		53.3	2.7	4.9	40.5	21.6		12.1					10.0	5.2
Lilium	0.3													
Loranthus					5.6	14.1							P	
Melilotus							34.3	25.7	21.6	13.6	85.8			
Myrtaceae**			8.7		18.3	33.1			1.6					
Trifolium pratense							1.6	3.2						11.8
Trifolium repens													44.7	13.0
Shrivelled pollen	10.4	7.3	7.0	9.1	19.5	13.2	14.7	8.7	11.5	9.8	13.2		10.0	8.6
Unidentified pollen	20.6	7.6	3.3	6.7	8.3	14.7	2.6	5.4	3.8	1.9	1.0		7.4	6.0
Total pollen grains counted	374	383	298	329	323	347	312	404	314	317	296	0*	351	347

For notes to key 1, 2 and 3 - refer to Table 31.

* - Sample examined for pollen but not present

** - Identified to plant family level only.

P - Pollen grain present but not counted.

TABLE 57 Cont'd.

Floral Genus	Pollen (%)[2,3] Honey sample code No[1]												
	207	208	209	210	211	212	213	214	215	216	217	218	219
Acacia							0.3						
Aesculus													79.8
Brassica		2.3			13.9		23.3		77.6	15.3	15.8	5.6	
Castanea										8.8	18.2		
Cirsium			11.8	7.2									
Echium					6.5		21.4						
Erica	1.2												
Fagopyrium			26.3		0.3								
Helianthus												64.9	5.4
Lilium							0.7						
Lotus		53.0	45.0				35.1						
Myrtaceae**								9.0					
Prunus/pyrus									8.1	6.8	4.3		
Robinia		3.8								9.1			
Taraxacum		4.3						5.0					
Tilia	1.2			1.5									
Trifolium pratense		10.9				1.3							
Trifolium repens				43.0		75.2				30.0	28.4	18.1	3.5
Urtica					68.5								
Vicia		1.3		0.4		3.9	17.4						
Viguirea	56.2							3.0					
Shrivelled pollen	11.8	18.8	15.1	23.0	5.2	9.8	8.5	17.4	8.1	20.7	26.7	8.5	9.5
Unidentified pollen	29.5	5.6	13.6	20.4	4.9	4.6	4.9	43.5	6.1	9.3	8.4	2.9	1.9
Total pollen grains counted	329	394	338	270	346	306	305	299	344	353	367	376	317

TABLE 57 Cont'd.

Floral Genus	Pollen (%)[2,3] Honey sample code No[1]													
	220	221	222	223	224	225	226	227	228	229	230	231	232	233
Acacia									1.7					
Banksia									0.8					
Brassica			15.1								2.0	8.8	28.0	19.4
Bruseraceae**								5.1	14.0					
Centaurea	7.3	8.8												
Cirsium										0.3		13.1		6.2
Cistus													2.9	
Echium												3.3		
Erica	10.9	11.0												
Eucalyptus									42.4		3.7	22.8		
Fagopyrum								6.4						
Helianthus			24.9										0.3	17.9
Lotus	30.8	41.9					17.4							
Melilotus							65.0							
Onobrychis	22.6													
Robinia			7.7					3.8						2.9
Rosmarinus							2.1							
Salix													47.5	
Taraxalum			2.4											
Tilia									1.7					
Trifolium pratense														4.8
Trifolium repens			14.2							79.2	59.0			8.4
Viguirea								31.0	6.9			6.7	3.2	
Shrivelled pollen	12.5	14.9	20.6				9.5	16.3	14.9	17.4	24.1	16.4	9.5	19.8
Unidentified pollen	15.8	23.4	15.1				6.0	37.4	17.5	3.0	11.3	28.9	8.7	20.5
Total pollen grains counted	367	308	337	0≠	0≠	0≠	380	393	349	361	407	329	379	273

≠ - Pollens present in these three samples but were not identifiable even at plant family level with the literature resources available.

whether the samples were commercially processed, that is, bottled or otherwise have been given in Table 58.

From the amino acid maps similar to that shown in Figure 7, the following ninhydrin positive substances were identified using reference standards in each honey sample within each of the sixteen country groups. These ninhydrin positive substances were: α- and β-alanine, α- and γ-amino-butyric acid, arginine, asparagine, aspartic acid, cysteine, glucosamine, glutamine, glutamic acid, glycine, histidine, hydroxypipecolic acid, hydroxyproline, isoleucine, leucine, lysine, methionine, methyl-histidine, phenylalanine, pipecolic acid, proline, serine, threonine, tryptophan, tyrosine, valine and the two unidentified spots 'x' and 'p'. Differences in occurrence of ninhydrin positive substances were observed in the amino acids maps and these were evaluated within and between each country group. These differences have been listed in Table 59. Moreover, there were other compounds which gave ninhydrin positive complexes whose identity could not be established using reference standards. Also these compounds were present in trace amounts which could not be eluted off from the cut-outs of the paper chromatograms for subsequent identification by mass spectroscopy. However, these compounds were labelled as ξ, δ, ζ, ε, π, ω, λ, θ, σ and Ω. The relative position of occurrence of these complexes to those identified have been shown in Figures 20 to 25.

Country of Origin Unknown, Suspect Origin and English Honey Types

Honey samples in this category were allocated into three groups and these were: samples whose country of origin was not stated on the bottle label or were obtained from comb of unspecified origin or were known by their floral names; samples whose identity of country source was suspected to be difference by their donors and samples of English

KEY - To Table 58

The key given below is also common to Table 61.

1. CNo = Code No., refer to Appendix I for country source.

2. CP = Commercially produced or otherwise.

T = Trace amounts.

Up = Raw hive honey or comb honey, or honey strained by cloth or processed by beekeeper or bulk honey from shipment container.

Pr = Commercially processed into bottles, jars, etc.

\+ = Information regarding the commercially processing or otherwise was not available.

TABLE 58

Free amino acids in known country type honeys

Amino acids	Concentration (nMoles per g of honey)										
CNo[1] →	193	194	195	196	197	198	199	200	201	202	203
Lys	T	80	200	141	104	136	78	162	143	162	158
Asp	315	140	151	70	111	85	73	97	50	172	73
Thr	143	138	210	100	122	202	24	105	96	254	91
Ser	120	158	145	44	92	129	38	91	55	116	95
Glu	277	206	142	107	125	226	107	140	74	177	142
Pro	4050	2000	2730	2370	2940	3140	2540	2720	1540	2710	2690
Gly	99	130	76	46	40	56	48	54	36	72	27
Ala	214	237	139	78	108	120	48	102	50	145	78
Val	61	52	85	60	78	123	27	76	26	122	46
Ile	34	30	67	36	62	77	13	55	27	150	30
Leu	23	76	312	220	73	91	14	70	21	158	29
Tyr	117	39	462	357	105	97	T	127	75	148	73
Phe	452	102	1370	910	980	510	56	540	58	102	150
CP[2]	U_p	P_r	U_p	+	U_p	U_p	Pr	+	+	+	+

Cont'd....

Table 58 cont'd.....

Amino acids	Concentration (nMoles per g of honey)									
	CNo[1] 204	205	206	207	208	209	210	211	212	213
Lys	51	59	81	T	85	140	177	160	520	46
Asp	105	193	154	102	78	230	136	130	131	73
Thr	23	41	38	33	48	261	92	75	83	33
Ser	47	80	90	46	37	220	102	86	88	67
Glu	105	211	154	207	52	150	131	110	114	66
Pro	3120	2210	2530	2710	2150	1490	2290	2240	1720	2630
Gly	36	59	54	43	34	208	170	96	148	72
Ala	64	91	83	79	46	293	252	139	268	146
Val	43	48	51	55	32	267	43	81	59	34
Ile	44	30	37	42	21	406	42	96	45	20
Leu	42	25	37	56	20	550	36	114	40	18
Tyr	T	61	44	45	56	356	110	109	100	T
Phe	122	132	400	416	33	700	850	700	870	86
CP	U_p	U_p	U_p	+	Yes	Yes	Yes	55	55	+

Cont'd....

Table 58 cont'd.....

Amino acids	Concentration (nMoles per g of honey)									
CNo[1] →	214	215	216	217	218	219	220	221	222	223
Lys	205	73	42	55	54	74	T	158	125	177
Asp	32	91	256	211	158	257	300	179	177	326
Thr	24	51	58	34	25	69	T	61	77	172
Ser	34	51	113	61	75	138	75	113	74	177
Glu	28	194	420	191	155	400	138	214	192	119
Pro	1620	1630	2990	2120	3590	6710	4800	3760	1900	1650
Gly	64	45	54	33	38	89	106	83	184	187
Ala	122	50	108	70	68	215	176	152	97	250
Val	20	42	91	31	21	77	42	38	57	106
Ile	11	27	34	19	31	54	30	40	41	69
Leu	14	25	20	T	T	41	14	26	32	84
Tyr	37	T	106	T	77	164	56	125	61	196
Phe	76	95	66	105	116	212	412	457	179	144
CP	Yes	Yes	Yes	Yes	Yes	Yes	Yes	+	Yes	U_p

Table 58 cont'd.....

Amino acids	Concentration (nMoles per g of honey)									
CNo[1] →	224	225	226	227	228	229	230	231	232	233
Lys	222	T	202	144	160	110	204	175	96	77
Asp	45	147	92	196	520	98	85	75	130	166
Thr	T	T	148	478	167	44	148	55	30	40
Ser	17	15	188	192	22	83	120	68	67	92
Glu	T	T	94	191	279	85	74	50	123	230
Pro	133	13	2980	2560	3500	4230	2510	2080	2500	2340
Gly	64	87	97	221	94	57	64	114	37	55
Ala	73	95	172	246	198	110	126	111	77	105
Val	T	T	77	133	119	48	60	52	53	45
Ile	T	T	49	108	75	29	49	30	22	35
Leu	T	T	54	132	65	22	48	39	24	26
Tyr	T	T	185	162	256	74	85	86	T	58
Phe	84	T	331	1230	1840	112	383	389	870	171
CP	Yes	Yes	Yes	Yes	U_p	Yes	U_p	U_p	U_p	+

TABLE 59

Occurrence of ninhydrin positive substances in known country honeys

Ninhydrin positive substances	Sample numbers in which given compound was found not* to occur
α-Abu	217, 218
γ-Abu	215, 217, 218, 225
β-Ala	196, 201, 202, 204, 206, 209, 212, 215, 217, 220, 221, 224, 225, 227, 228, 229
Arg	193, 196, 205, 206, 207, 210, 212, 213, 214, 215, 216, 217, 218, 220, 224, 225, 228, 229, 231, 232, 233
Asn	201, 202, 204, 209, 212, 215, 221
Cys	196, 197, 201, 202, 204, 208, 220, 221, 226, 227, 231
Gluc	216, 232
Gln	199, 200, 201, 202, 204, 208, 224, 227, 232
His	218, 224, 225
H-pip	193, 199, 201, 202, 203, 204, 205, 207, 208, 209, 211, 212, 213, 214, 215, 216, 217, 218, 219, 222, 229, 232, 233
Me-His	204, 208, 219, 222, 226
Pip	201, 204, 210, 212, 214, 215, 216, 217, 220, 224, 225, 232
Trp	201, 204, 208, 215, 216, 217
'x'	217, 118
'p'	193, 195, 197, 198

In addition to the other amino acids found in all samples, the following ninnydrin positive substances were also present. These were: hydroxyproline in sample numbers 197, 222 and 223; the unidentified spot 'x' in sample numbers 193, 206, 213, 214, 223; the unidentified spot 'p' in sample numbers 199, 201, 208, 224 and 225; the unidentified spot 'ε' in sample numbers 211, 222 and 223; the unidentified spot 'Ω' in sample numbers 223, 225 and 231; the unidentified spot 'ξ' in sample numbers 209, 210 and 212; the unidentified spot 'δ' in sample numbers 209 and 211; the unidentified spot 'σ' and 'w' in sample number 209; the unidentified spot 'λ' in sample number 211 and the unidentified spot 'θ' in sample number 214.

* Details as per Table 33.

FIGURE 20

Amino acid map of honey from Australia No. 194 - *Banksia*.

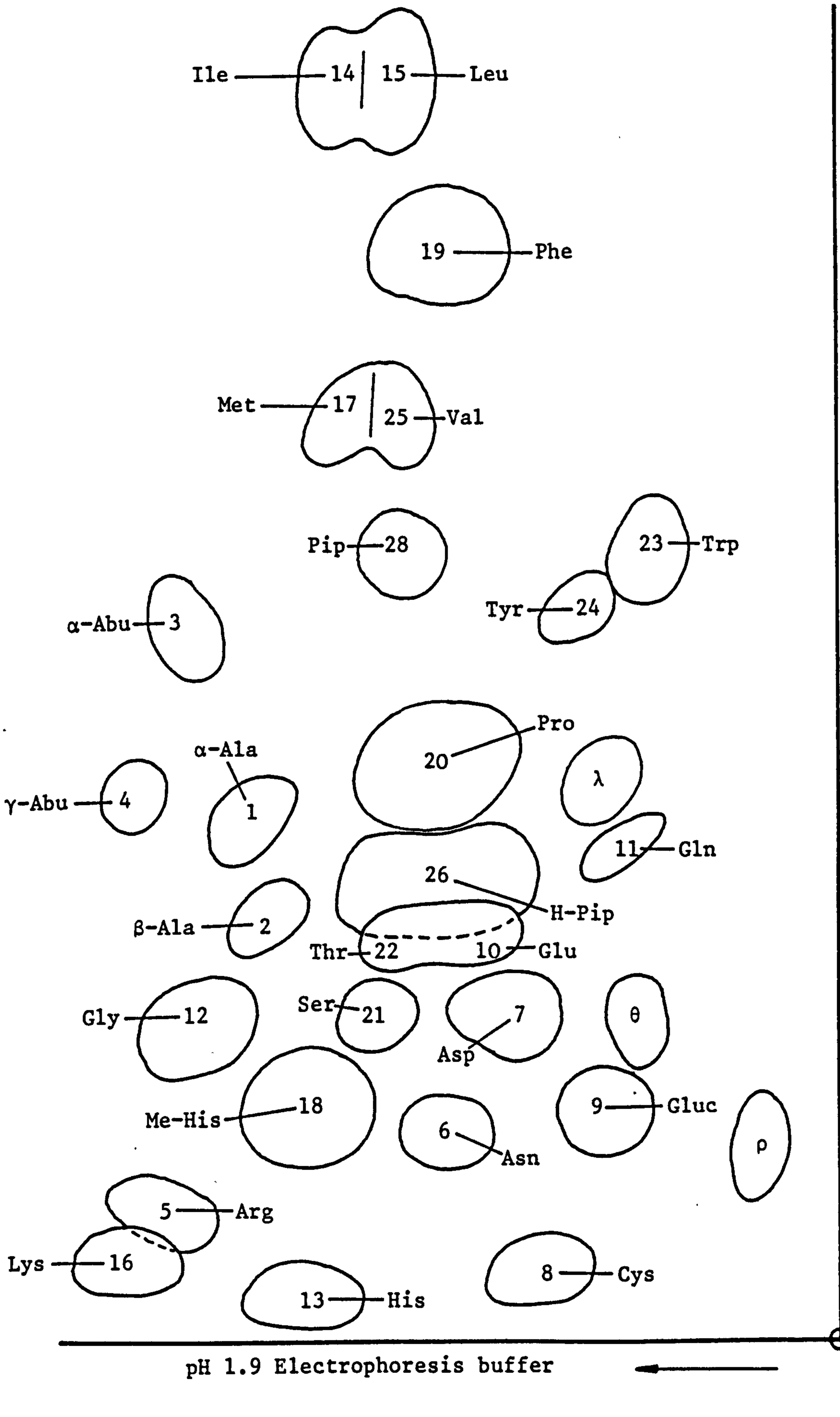


FIGURE 21.

Amino acid map of honey from Canada No. 199

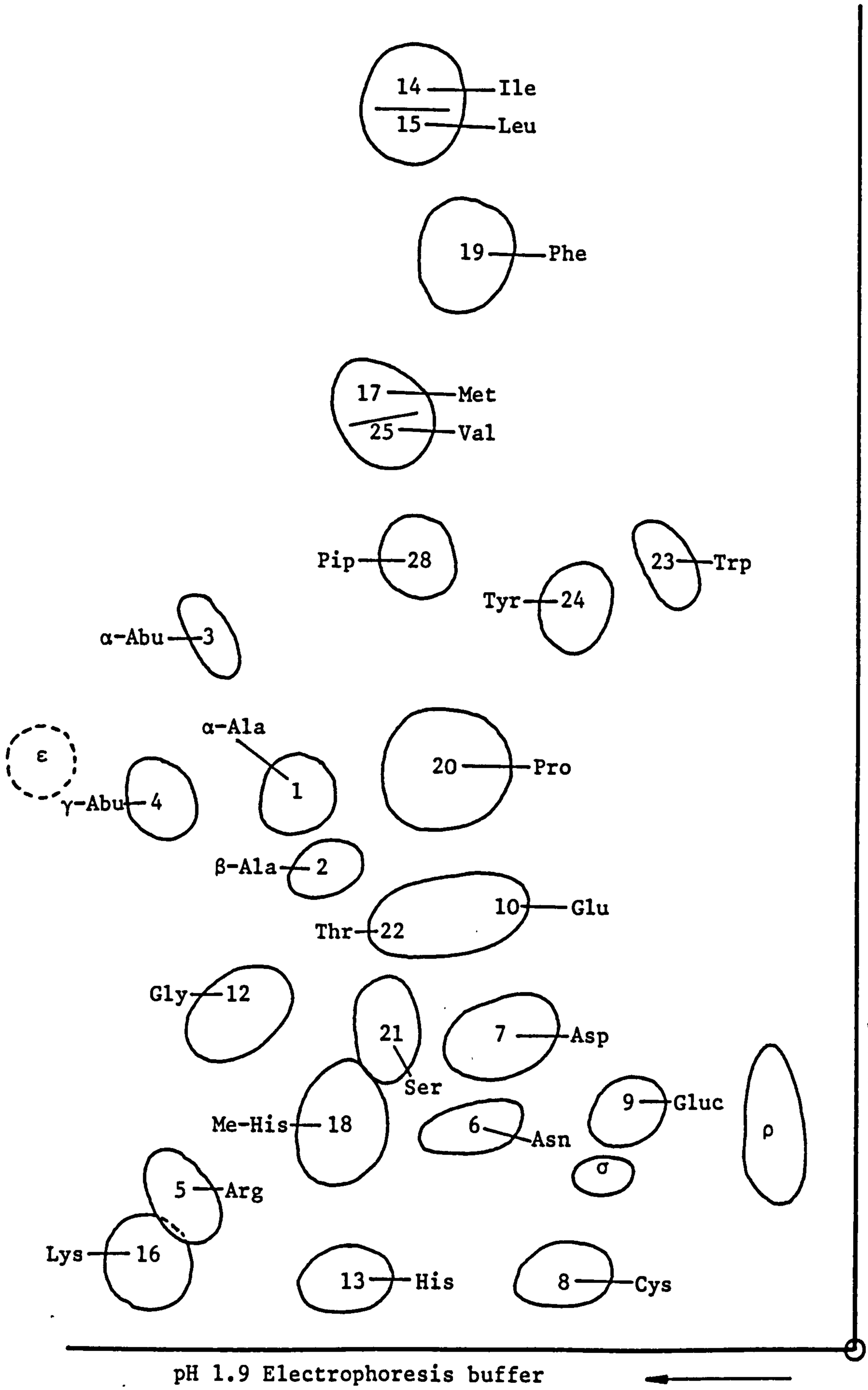


FIGURE 22

Amino acid map of honey from China No. 209 - Buckwheat.

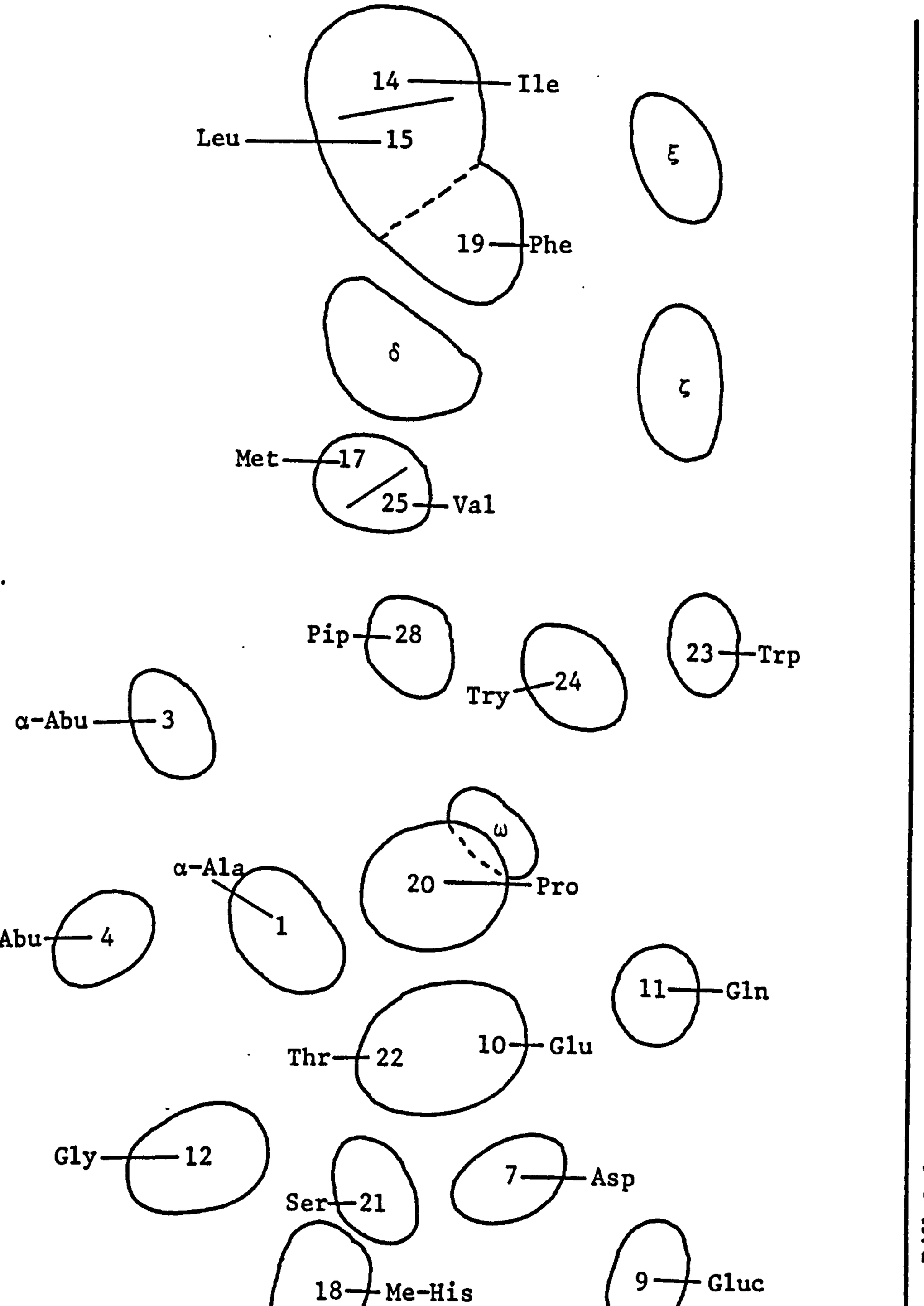


FIGURE 23

Amino acid map of honey from India No. 225 - Gujarat 2.

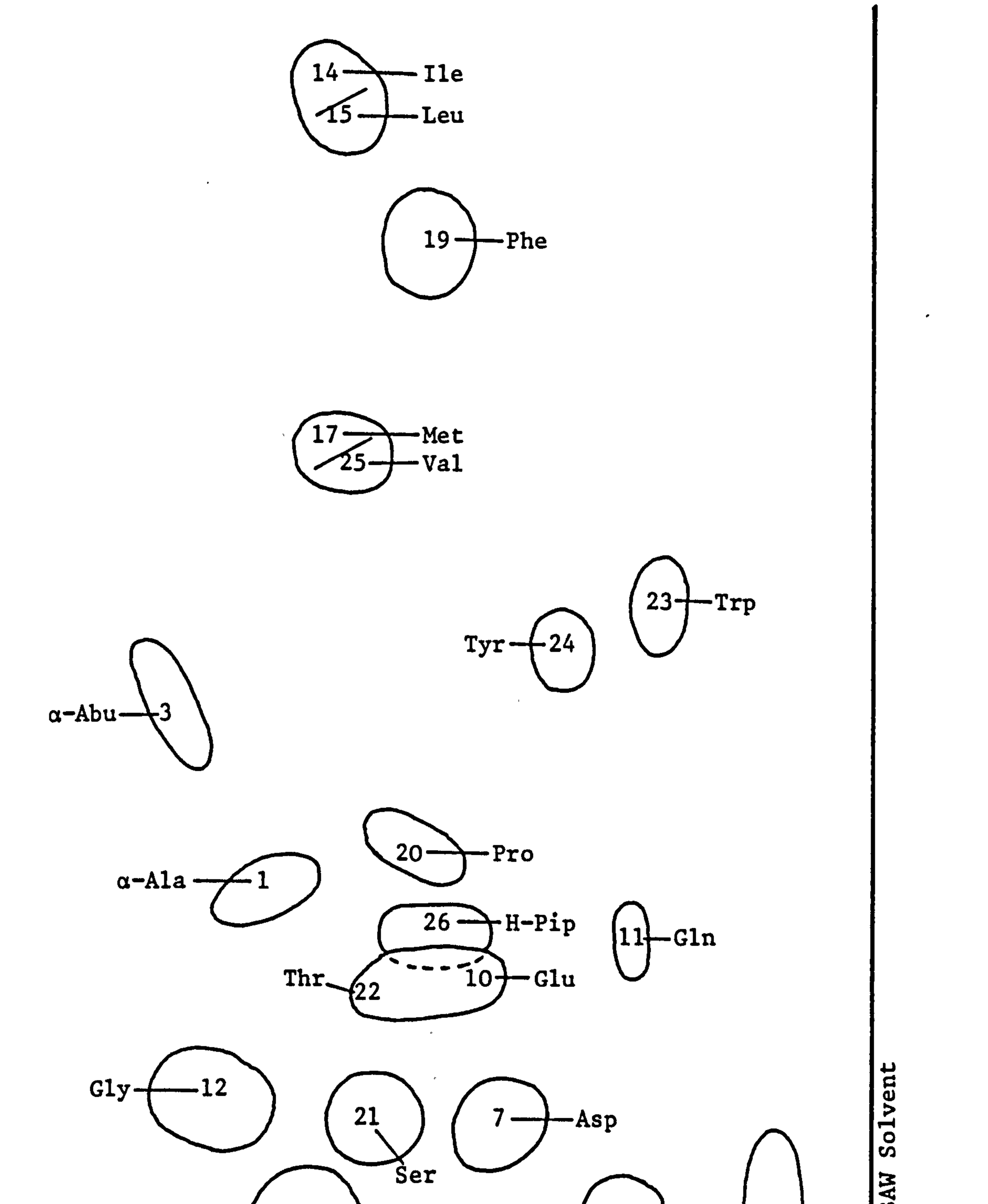


FIGURE 24

Amino acid map of honey from Mexico No. 227 - Yucatan.

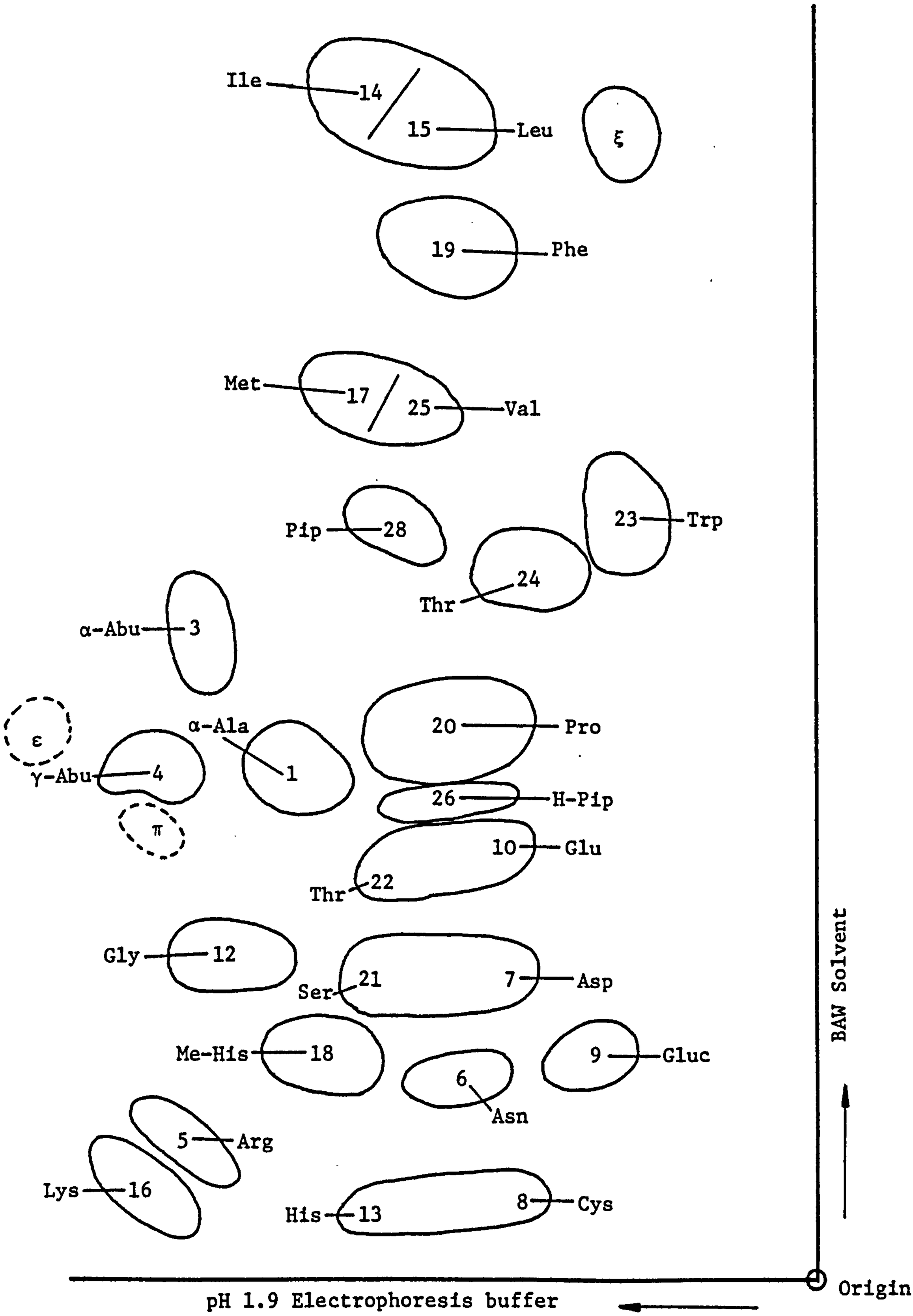
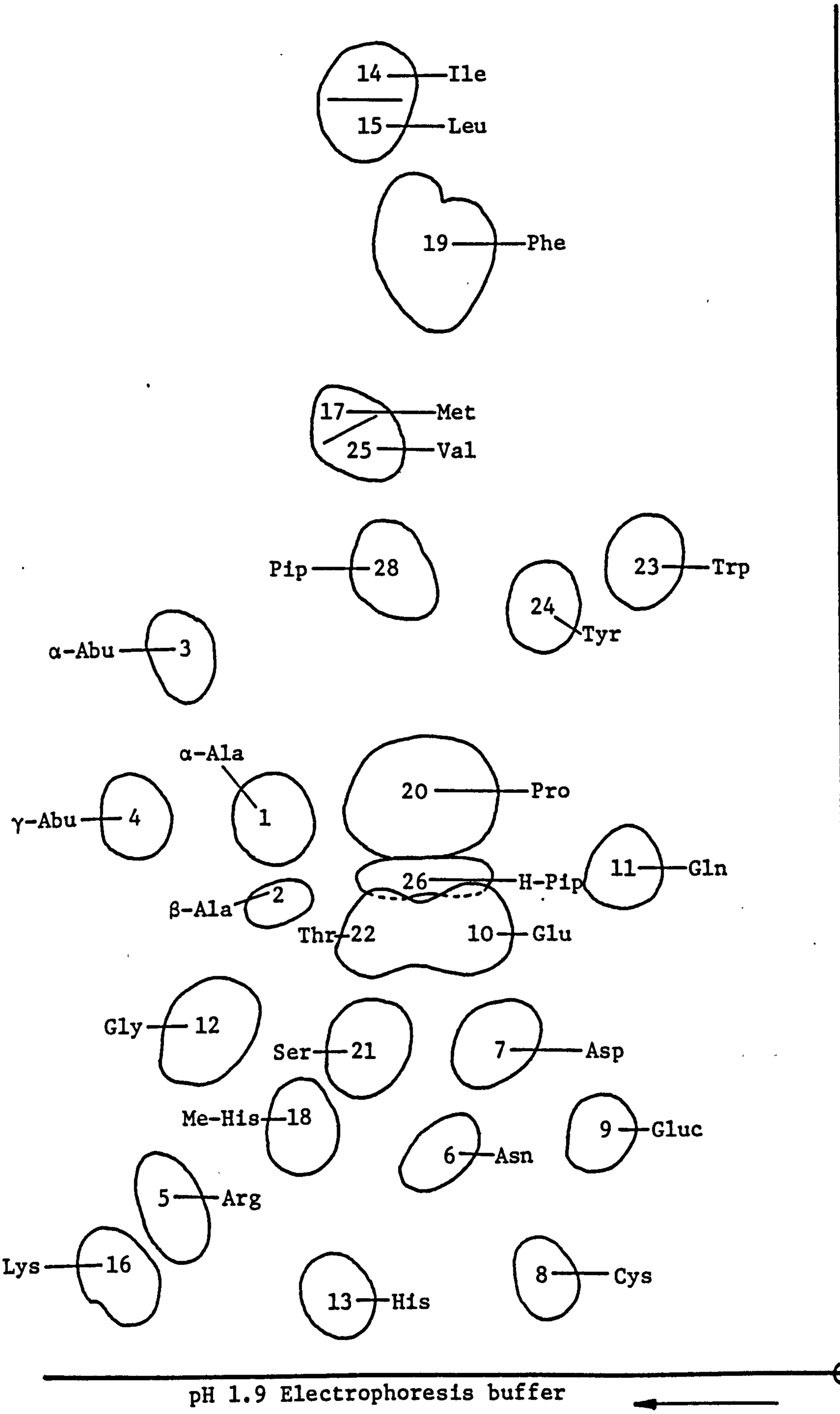


FIGURE 25

Amino acid map of honey from New Zealand No. 230 - Clover.



honey that were processed by the beekeeper or by commercial producers. There were twenty one honey samples that accounted for these three groups. The pollen content of each sample of honey in the three groups have been given in Table 60. The amino acid concentration measurements and the information whether the samples were commercially processed, that is, bottled or otherwise have been given in Table 61.

The following ninhydrin positive substances were identified on the amino acid maps using reference standards in each of the honey samples within each of the three groups. These ninhydrin positive substances were: α- and β-alanine, α- and γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, glucosamine, glutamine, glutamic acid, glycine, histidine, hydroxypipecolic acid, isoleucine, leucine, lysine, methionine, methyl-histidine, phenylalanine, pipecolic acid, proline, serine, threonine, tryptophan, tyrosine and valine. The differences in occurrence of the ninhydrin positive substances on the amino acid maps were evaluated wihin and between each of the three groups. These differences have been given in Table 62.

Sugar Product Type

Commercially refined sugar products were allocated into this category and accounted for two samples, one sample Code No. 255 was derived from potato starch and was being sold as man made honey. The other sample Code No. 256 was a standard sugar syrup sold at various commercial retailers. These sugar samples did not contain pollen. The amino acid concentration measurements have been given in Table 61.

There were no ninhydrin positive substances detected on the replicate amino acid maps of sample Code No. 255. The following ninhydrin positive substances were detected and identified on the replicate amino acid maps of the sugar syrup sample Code No. 256 using reference standards. These

TABLE 60

Frequency of distribution of pollen grains in country of origin now known, suspect origin, and English honeys and sugar products.

Floral Genus	Pollen (%)[2,3] Honey sample code No[1]										
	234	235	236	237	238	239	240	241	242	246	247
Acacia					1.0				1.5		
Banksia				0.6	4.5	1.9			1.2		
Brassica				20.5	19.4	16.8	17.2	10.2	21.5		
Castanea											61.2
Echium				5.9	10.0	14.9		57.2	37.1	65.0	
Eucalyptus				13.4	35.4	40.5	4.5	4.5	12.4	5.3	
Helianthus								1.1			
Loranthus							0.5			4.6	
Lotus				14.6	6.3		14.6				
Myrtaccae**						5.5			5.8	4.1	
Robinia				14.0			19.9	8.0			
Rosmarinus											12.5
Taraxacum							7.2	1.9			
Trifolium repens									2.4		15.6
Urtica							8.2	1.3			
Shrivelled pollen				16.7	14.4	12.7	16.0	12.9	11.9	20.2	10.1
Unidentified pollen				14.3	8.9	7.7	11.9	8.8	6.2	0.8	0.6
Total pollen grains counted	0*	0*	0*	336	381	363	377	373	410	392	327

For notes to key 1, 2 and 3 - refer to Table 31.

* - Sample examined for pollen but none present, it is suspected that these three samples were subjected to high pressure filtration for the purpose of clarification.

** - Identified at plant family level only.

TABLE 60 Cont'd.

Floral Genus	Pollen (%)[2,3] Honey sample code No.[1]											
	243	244	245	253	254	248	249	250	251	252	255	256
Brassica	32.4	17.9	10.8	39.7	74.0	24.6			74.8			
Calluna								3.1	9.0	7.9		
Castanea	11.8	21.5	11.8			12.1				68.7		
Echium	6.0	16.2		0.6		5.1						
Epilobium								1.2				
Eucalyptus	3.6	8.8		6.9		6.0						
Heracleum	4.7	1.3	3.0									
Impatiens								2.2				
Ligustrum								56.7				
Loranthus				2.9		1.9						
Lotus				5.8								
Onobrychis	3.0	0.7	8.6									
Phlox								0.7				
Prunus/pyrus					6.2				1.5			
Robinia						3.7						
Rosmarinus	0.8					0.5						
Tilia							2.5					
Trifolium pratense						2.5						
Trifolium repens	15.4	14.6	35.8	17.7	0.5		62.9	12.7		14.5		
Vicia					4.2							
Shrivelled pollen	13.5	12.9	21.1	21.1	10.1	23.7	28.5	18.7	10.2	7.5		
Unidentified pollen	8.8	6.1	8.8	5.8	6.0	19.9	6.1	4.6	9.5	0.7		
Total pollen grains counted	364	396	397	345	385	430	256	236	322	428	0+	0+

+ - Sample examined for pollen, but none present.

TABLE 61

Free amino acids in unknown country of origin, suspect origin and English honey and sugar products

Amino acids	Concentration (nMoles per g of honey)										
	CNo[1] 234	235	236	237	238	239	240	241	242	246	247
Lys	51	19	25	64	45	85	44	T	54	230	82
Asp	202	176	161	84	93	72	83	49	109	62	83
Thr	89	60	58	29	46	106	27	17	31	106	44
Ser	104	92	93	65	69	69	55	33	53	47	73
Glu	180	180	153	77	116	95	69	53	85	107	158
Pro	3050	2920	3170	2080	2480	2760	2010	2310	2430	2860	6510
Gly	73	64	57	59	70	69	50	38	69	41	47
Ala	115	110	101	61	93	100	52	38	80	79	129
Val	107	66	47	41	37	68	24	26	40	67	77
Ile	93	55	46	21	24	43	22	17	35	67	42
Leu	112	58	65	14	28	59	17	46	90	468	21
Tyr	260	130	139	T	90	91	87	342	170	550	306
Phe	1780	710	460	52	301	490	111	1610	620	1260	1550
CP	U_p	U_p	U_p	U_p	U_p	U_p	U_p	U_p	U_p	P_r	P_r

Cont'd....

Notes to key 1, 2, T, U_p , P_r and +, refer to fornote to Table 58.

Table 61 cont'd.....

Amino acids	Concentration (nMoles per g of honey)											
CNo[1] →	243	244	245	253	254	248	249	250	251	252	255	256
Lys	423	240	271	41	37	27	67	38	87	21	-	-
Asp	94	91	63	96	90	113	41	81	96	67	83	73
Thr	234	137	123	30	41	54	18	31	65	18	-	-
Ser	112	111	83	54	59	76	42	52	93	41	T	48
Glu	98	78	107	102	89	143	61	81	231	81	-	T
Pro	2520	2370	2220	2240	1760	2870	3220	2930	2780	1840	-	-
Gly	57	57	81	61	56	60	67	42	70	57	87	20
Ala	107	92	97	77	76	98	55	78	118	93	50	80
Val	91	74	57	35	51	65	32	41	71	28	-	T
Ile	87	71	70	34	31	46	23	530	44	12	T	19
Leu	100	107	94	32	30	41	18	297	42	T	-	-
Tyr	169	146	103	85	T	109	66	60	1250	125	-	-
Phe	840	720	232	450	157	800	47	136	1970	40	-	-
CP	U_p	U_p	U_p	U_p	U_p	U_p	P_r	P_r	U_p	+	P_r	P_r

TABLE 62

Occurrence of ninhydrin positive substances in unknown country, suspect origin and English honeys

Ninhydrin positive substances	Sample numbers in which given compound was found not* to occur
α-Abu	241, 248
γ-Abu	248
β-Ala	237, 240, 241, 243, 244, 247, 248, 249, 251
Arg	235, 236, 237, 238, 239, 240, 241, 242, 248, 249, 251, 252, 253, 254
Asn	245, 249, 252
Cys	237, 245, 248
Gluc	250
Gln	245
H-pip	234, 237, 240, 241, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253
Me-His	234, 246, 248
Pip	237, 239, 240, 241, 242, 244, 246, 247, 248, 249, 250, 251, 252
'x'	234, 235, 237, 238, 240, 241, 246, 247

In addition to the other amino acids found in all samples, the following ninhydrin positive substances were also present. These were: the unidentified spot 'p' in sample number 234; the unidentified spot 'λ' in sample number 246 and the unidentified spot 'ε' in sample numbers 243 and 245.

* Details as per Table 33.

ninhydrin positive substances were: α-alanine, α-aminobutyric acid, aspartic acid, asparagine, glycine, isoleucine, leucine, methyl-histidine, methionine, serine, threonine and valine. The amino acid map for sample Code No. 256 has been illustrated in Figure 26.

The relative concentration of the amino acids in these sugar products were much lower than those compared to the honey sample.

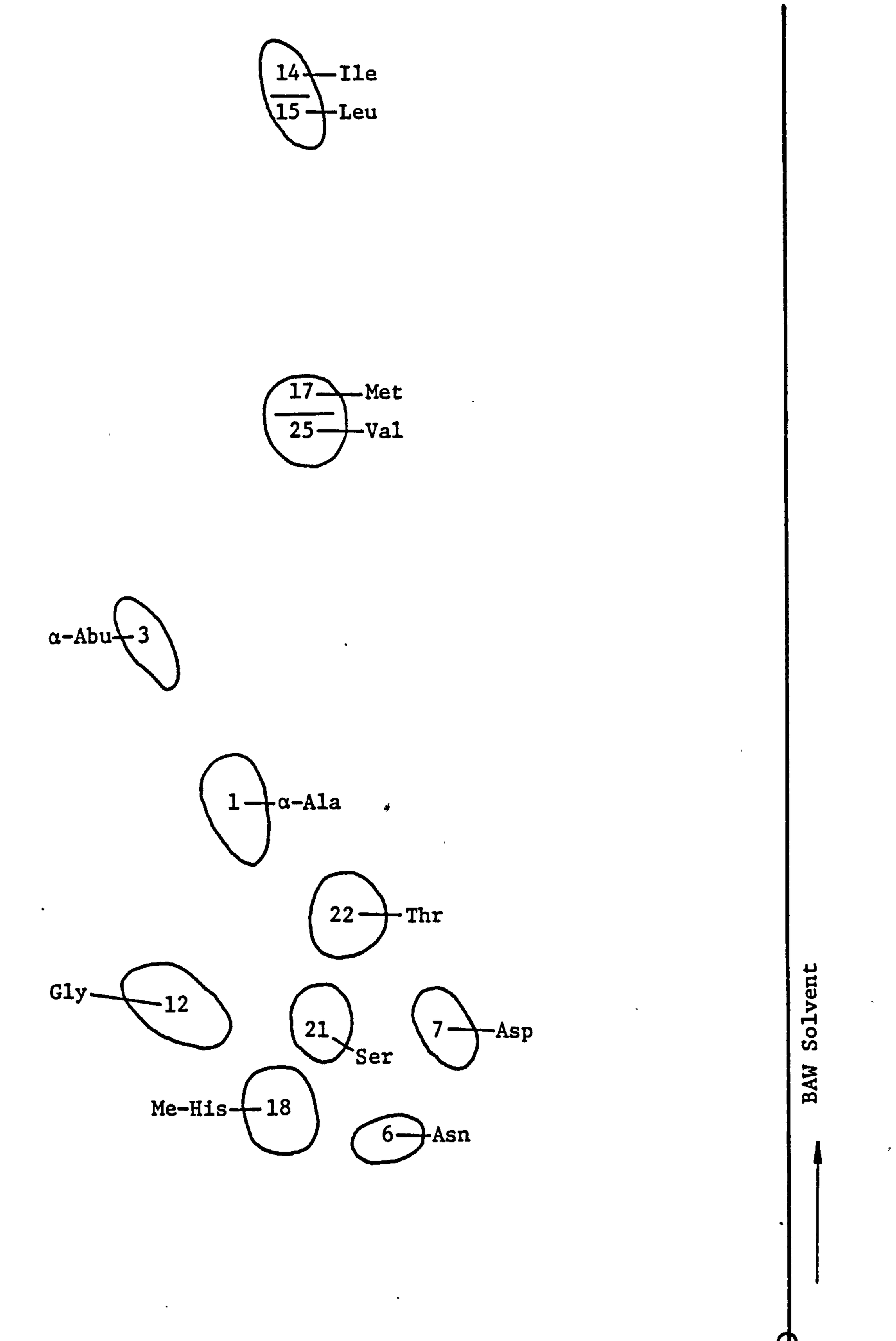
Computer Processing of Analytical Data

In evaluating, allocating and classification of the data given in Tables 31 to 62 for each of the two hundred and fifty six honey samples into pre-selected groups on the basis of the amino acid analytical data necessitated the use of a complex statistical procedure. It was considered preferable to utilize a computer with the appropriate software package. The statistical package for social sciences (SPSS) on the PRIME computer was used for performing 'DISCRIMINANT ANALYSIS' using the statistical 'MAHAL' method. This statistical 'MAHAL' method has been defined and described in Appendix VI(a). In the following text, tables and figures the statistical package will be referred to by the abbreviation SPSS.

The standards used for evaluating the analytical data of the amino acids were randomly selected by the SPSS from the available information. The standards selected by the SPSS were 'reference standards'; in default of officially recognised reference standards. As a consequence of computer programming standardised responses using the given data were obtained. In some cases the SPSS programme gave a misclassification in respect to the experimentally observed status. However, in most cases of misclassification there were some agreement as to selected group or the criteria. Moreover, when any such results of misclassification became available the experimental pollen analyses were re-examined in conjunction

FIGURE 26

Amino acid map of Sainsbury's golden syrup No. 256.



with the paper electrophoresis and chromatography amino acid patterns, which were not given to the computer becuase of their qualitative nature. The experimental origins take account of any such reassessment and are as stated.

The merits of this SPSS predictive classification and the present dangers of misclassification by the SPSS have been dealt with in the discussion.

Analyses of Samples from the United Kingdom Survey Honeys

Computer placements of honey samples into pollen groups

Honey samples of the predominant pollen type were selected for discriminant analysis using the specifications listed in control statements 55 to 63 of the SPSS control file given in Appendix IV(b). A selected range of amino acid concentration measurements were allocated an arbitary number so as to reduce the number of variables processed by the SPSS as detailed in Appendix VI(b). The predicted classification of the predominant pollen type honey samples into nine groups by the SPSS have been given in Table 63. These samples were classified on the basis of the amino acids for which the Fisher's linear discriminant functions were calculated and then these amino acids were entered into the discriminant analysis. These amino acids were: aspartic acid, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, phenylalanine and a constant derived by the SPSS. The SPSS selected one hundred and four honey samples and of these only 45.2% were correctly classified into their respective pollen groups.

The amino acid measurements were modified as described and the pollen groups containing inadequate sample numbers were ommited from analysis. Hence, five pollen groups thought to contain adequate sample numbers

TABLE 63

Classification of the U.K. honey samples into nine pollen groups by the SPSS

Pollen groups	No. of Samples	SPSS predicted pollen group membership								
		301*	308*	318*	319*	328*	305*	304*	309*	326*
301*	40	15	1	1	14	4	1	0	1	3
308*	28	11	16	3	0	2	4	1	0	1
318*	17	1	4	4	0	2	1	0	0	5
319*	6	1	0	1	4	0	0	0	0	0
328*	8	1	0	1	0	4	1	0	0	1
305*	2	0	0	1	0	0	1	0	0	0
304*	1	0	0	0	0	0	0	1	0	0
309*	1	0	0	0	0	0	0	0	1	0
326*	1	0	0	0	0	0	0	0	0	1

* = Refer to Table 30 for pollen name.

were examined. The unifloral and multifloral subclasses were determined for each of the four pollen groups namely *Brassica*, *Trifolium repens*, *Castanea* and *Calluna*, as detailed previously. The fifth pollen group, *Myosotis* did not require sub-classification since the honey samples were predominantly unifloral being greater than 95% pollen of one species. The results of the computer classification of honey samples into each subclass for the above pollen groups were found to be similar. However, the point at which the overlap between the two subclasses was eliminated, varied for each of the pollen groups. These points were: *Brassica* group at 80% and over, for *Trifolium repens* and *Castanea* groups at 75% and over, and for *Calluna* group at 70% and over. As a typical example the results obtained in the form of a histogram for the *Brassica* group has been shown in Figure 27. The results of the classification for the ungrouped honey samples by the SPSS have also been shown. The peaks of the two subclasses for the ungrouped samples follow a similar pattern to that of the *Brassica* samples. Thus, having defined the unifloral subclass as those samples containing 70% pollen and over for the four pollen groups.

The classification of the honey samples into the five pollen groups was carried out using the modified SPSS control file given in Appendix VI(c). The honey samples in each of the five pollen groups were divided into two subsets by the SPSS. In one subset of honey samples the Fisher's linear discriminant functions were calculated for the following amino acids which were entered into the analysis. These were: proline, glycine, valine, isoleucine and a constant derived by the SPSS. The SPSS selected twenty nine honey samples in this subset and of these only 89.7% were correctly classified into their respective pollen groups. These results have been shown in Table 64.

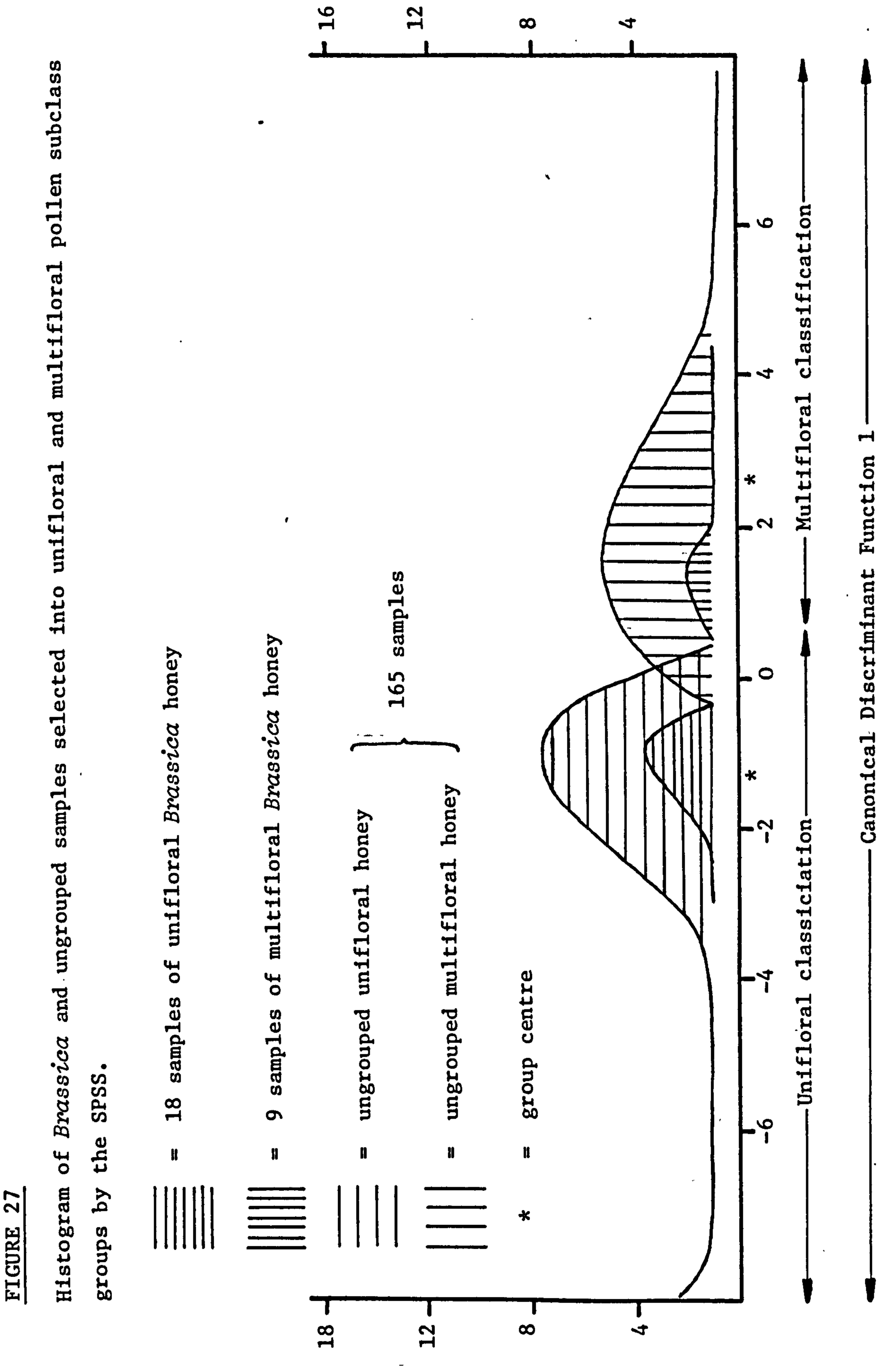

Histogram of unifloral and multifloral pollen subclass groups by the SPSS.
= 18 samples of unifloral Brassica honey
= 9 samples of multifloral Brassica honey
= ungrouped unifloral honey
= ungrouped multifloral honey
165 samples
= group centre
Unifloral classification
Multifloral classification
Canonical Discriminant Function 1
Frequency
16
12
8
4
6
4
2
0
-2
-4
-6

FIGURE 27

TABLE 64

Classification of the U.K. honey samples into five pollen groups by the SPSS

Pollen groups	No. of samples	SPSS predicted pollen group membership				
		301*	308*	318*	319*	328*
A						
301*	17	17	0	0	0	0
308*	1	0	1	0	0	0
318*	6	1	0	3	0	2
319*	2	0	0	0	2	0
328*	3	0	0	0	0	3
B						
301*	12	11	0	1	0	0
308*	2	0	1	1	0	0
318*	3	0	0	0	1	2
319*	3	1	0	0	1	1
328*	4	1	1	2	0	0

* = Refer to Table 30.

A = Samples used for discriminant function calculation.

B = Samples used for estimating misclassification rate.

There were six honey samples selected in the *Castanea* group and the SPSS misclassified three samples. The SPSS correctly predicted the presence of *Brassica* pollen in sample Code No. 89 but was incorrect for the samples Code No. 28 and 36.

In the other subset of honey samples, twenty four samples were used by the SPSS for estimating the misclassification rate and of these only 54.2% of the samples were correctly classified into their respective pollen groups.

There were eleven samples misclassified in this subset and the SPSS correctly predicted the presence of the pollen in eight samples Code Nos. 39, 41, 102, 106, 118, 121, 171 and 177, for which these samples were predicted to contain. The remaining three samples Code Nos. 57, 84 and 91 did not agree as to the pollen predicted to be present.

Graphical representation of the classification of the honey samples has been shown in Figure 28. This scatterplot of the canonical discriminant function 1 against canonical discriminant function 2 was derived by the SPSS. The samples identified above as misclassified have been outlined.

The computer classification of honey samples into the three major pollens identified in the present survey of the United Kingdom have been given in Table 65. The SPSS selected eighteen samples of honey for calculating the Fisher's linear discriminant functions and the following amino acids were entered into the analysis. These were: lysine, aspartic acid, threonine, glutamic acid, proline, glycine, alanine, isoleucine, leucine, tyrosine and a constant derived by the SPSS. The honey samples were all 100.0% correctly classified into their respective pollen groups.

The SPSS then selected nineteen samples for which it estimated the misclassifiation rate and of these only 68.4% were correctly classified

FIGURE 28

The five pollen group scatterplot, by the SPSS.

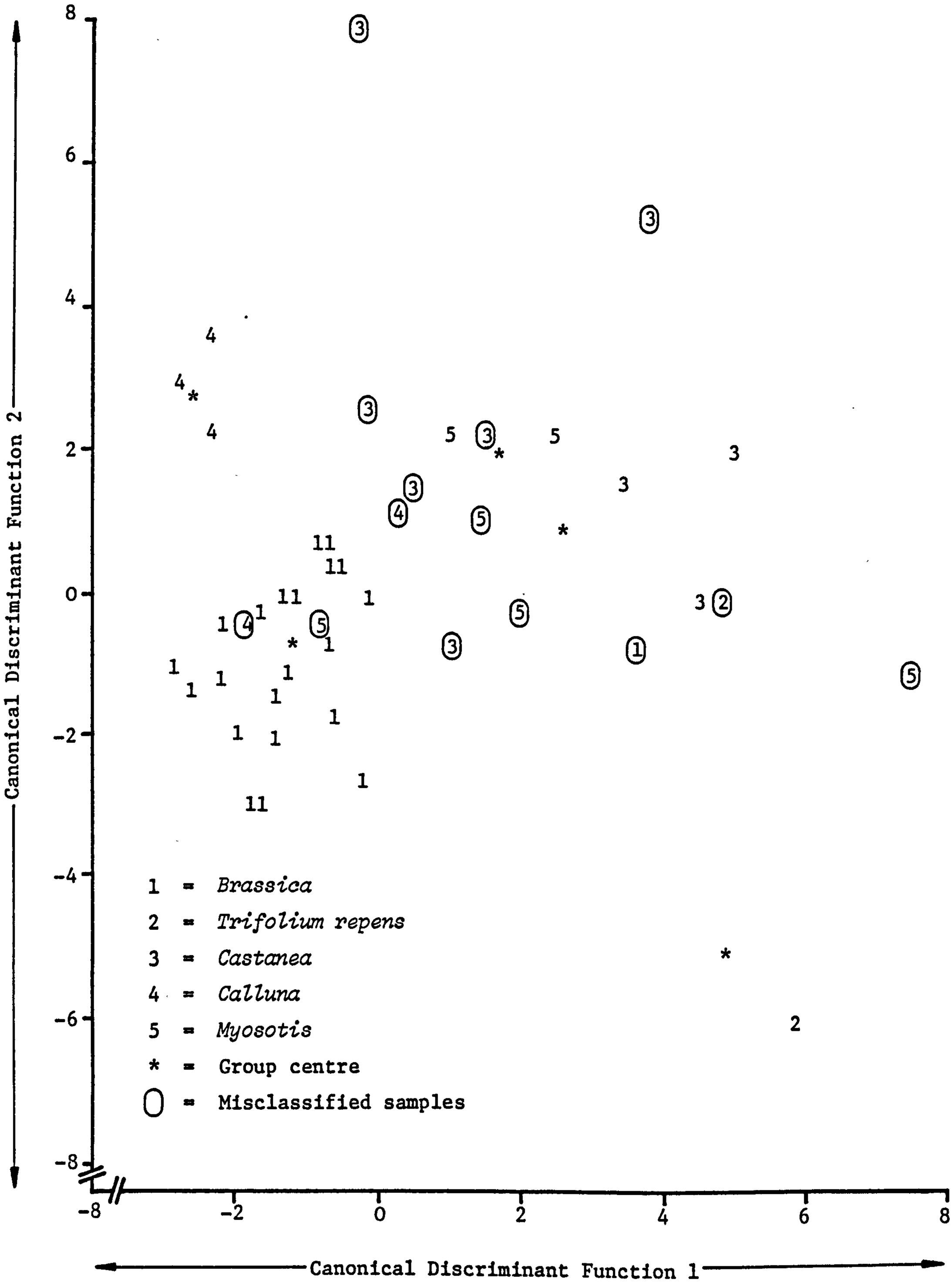


TABLE 65

Classification of the U.K. honey samples into three pollen groups by the SPSS

Pollen groups	No. of samples	SPSS predicted pollen group membership		
		301*	308*	319*
A				
301*	15	15	0	0
308*	1	0	1	0
319*	2	0	0	2
B				
301*	14	10	1	3
308*	2	0	2	0
319*	3	2	0	1

TABLE 66

Classification of the U.K. honey samples into the 1981 and 1982 calendar year groups by the SPSS

Year groups	No. of samples	SPSS predicted year group membership		Percent of group correctly classified by SPSS
		1981	1982	
A				
1981	33	30	3	83.3%
1982	39	9	30	
B				
1981	33	24	9	65.6%
1982	63	24	39	

These notes are common to both Tables 65 and 66:

* = Refer to Table 30.

A = Refer to Table 64.

B = Refer to Table 64.

into their respective pollen groups.

There were six samples misclassified in this subset and the SPSS correctly predicted the presence of the pollen in three samples Code Nos. 118, 147 and 152 for which these samples were predicted to contain. The remaining three samples Code Nos. 66, 115 and 145 did not agree as to the pollen predicted to be present.

Computer placements of honey samples into 1981 and 1982 calender groups

In accordance with the responses recieved to questionnaire question number 3 the SPSS was specified as described to allocate honey samples into the two calender year groups. The predicted classification of one hundred and sixty eight selected samples have been given in Table 66. The Fisher's linear discriminant functions were calculated for the following amino acids which were entered into the analysis. These were: lysine, aspartic acid, threonine, proline, alanine, valine and a constant derived by the SPSS.

Using the control statements given in Appendix VI part (dii) 1, 2, 3, 4 and 5 representing the five pollen groups, the SPSS allocated samples which satisfied these criteria for each of the pollen groups into the two calendar year groups. The results were obtained for only three of the pollen groups and these were *Brassica*, *Trifolium repens* and *Castanea*. The predicted classification of the seventy five honey samples selected by the SPSS have been given in Table 67.

The amino acids for which the Fisher's linear discriminant functions were calculated differed in these three pollen groups. These differences were: in the *Brassica* group, the amino acids used were valine, isoleucine, tyrosine, phenylalanine and a constant derived by the SPSS; in the

TABLE 67

Classification of the U.K. honey samples representing the three pollens into 1981 and 1982 calendar year groups by the SPSS

Year groups	No. of samples	SPSS predicted year group membership		Percent of group correctly classified by SPSS
		1981	1982	
Brassica				
A				
1981	6	5	1	77.3%
1982	16	4	12	
B				
1981	2	0	2	47.1%
1982	15	7	8	
Trifolium repens				
A				
1981	8	8	0	100.0%
1982	5	0	5	
B				
1981	2	1	1	62.5%
1982	6	2	4	
Castanea				
A				
1981	7	7	0	100.0%
1982	1	0	1	
B				
1981	4	2	2	28.6%
1982	3	3	0	

A = Refer to Table 64.

B = Refer to Table 64.

Trifolium repens group, the amino acids used were lysine, serine, proline, glycine, alanine, leucine, tyrosine and a constant derived by the SPSS and in the *Castanea* group, the amino acids used were lysine, aspartic acid, threonine, proline, valine, tyrosine and a constant derived by the SPSS.

No distinct pattern was noted in the samples that were misclassified in Tables 66 and 67.

Computer placements of honey samples which were heated or otherwise

In accordance with the responses received to questionnaire question number 5 the SPSS was specified to allocate samples into the 'heated' and 'not heated' groups as described. The predicted classification of one hundred and seventy one honey samples selected by the SPSS have been given in Table 68. The Fisher's linear discriminant functions were calculated for the following amino acids which were entered into the analysis. These were: threonine, serine, glycine, phenylalanine and a constant derived by the SPSS.

The specifications for the 'heated' and 'not heated' groups were substituted for the two calendar year groups as given in Appendix VI part (dii) 1, 2, 3, 4, and 5 representing the five pollen groups. The SPSS allocated samples which satisfied these criteria for each of the pollen groups into the 'heated' and 'not heated' groups. The results were obtained for only three pollen groups and these were *Brassica*, *Castanea* and *Myosotis*. The predicted classification of the sixty four honey samples selected by the SPSS have been given in Table 69. The amino acids for which the Fisher's linear discriminant functions were calculated differed in each of the three pollen groups. These differences were: in the

TABLE 68

Classification of the U.K. honey samples into 'heated' and 'not heated' groups by the SPSS

Heat groups	No. of samples	SPSS predicted heat group membership		Percent of group correctly classified by SPSS
		heated	not heated	
A				
heated	17	10	7	72.3%
not heated	77	19	58	
B				
heated	15	4	11	66.2%
not heated	62	15	47	

TABLE 69

Classification of the U.K. honey samples representing three pollen groups into 'heated' and 'not heated' groups by the SPSS

Heat groups	No. of samples	SPSS predicted heat group membership		Percent of group correctly classified by SPSS
		heated	not heated	
Brassica				
A				
heated	7	7	0	94.7%
not heated	12	1	11	
B				
heated	3	1	2	55.0%
not heated	17	7	10	
Castanea				
A				
heated	1	1	0	100.0%
not heated	8	0	8	
B				
heated	2	1	1	75.0%
not heated	6	1	5	
Myosotis				
A				
heated	1	1	0	100.0%
not heated	5	0	5	
B				
heated	0	0	0	100.0%
not heated	2	0	2	

These notes are common to both Tables 68 and 69.

A = Refer to Table 64.

B = Refer to Table 64.

Brassica group, the amino acids used were lysine, serine, proline, leucine, phenylalanine and a constant derived by the SPSS; in the *Castanea* group, the amino acids used were aspartic acid, threonine, glutamic acid, valine, isoleucine, tyrosine and a constant derived by the SPSS and in the *Myosotis* group, the amino acids used was glutamic acid and a constant derived by the SPSS.

Computer placements of honey samples which were a result of sugar feeding or otherwise

In accordance with the responses received to questionnaire question number 6 the SPSS was specified to allocated honey samples into the 'sugar-fed' and the 'not sugar-fed' groups as described. The predicted classification of the one hundred and seventy one honey samples selected by the SPSS have been given in Table 70. The Fisher's linear discriminant functions were calcualted for the following amino acids which were entered into the analysis. These were: lysine, threonine, isoleucine and a constant derived by the SPSS.

The specifications for the 'sugar-fed' and 'not sugar-fed' groups were substituted for the two calendar year groups as given in Appendix VI part (dii) 1, 2, 3, 4 and 5 representing the five pollen groups. The SPSS allocated samples which satisfied these criteria for each of the pollen groups into the 'sugar-fed' and 'not sugar-fed' groups. The predicted classification of the ninety five honey samples selected by the SPSS have been given in Table 71.

The amino acids for which the Fisher's linear discriminant functions were calculated differed in the five pollen groups. These differences were: in the *Brassica* group, the amino acids used were lysine, serine,

TABLE 70

Classification of the U.K. honey samples into 'sugar-fed' and 'not sugar-fed' groups by the SPSS

Sugar groups	No. of samples	SPSS predicted sugar group membership		Percent of group correctly classified by SPSS
		sugar-fed	not sugar-fed	
A				
sugar-fed	61	37	24	64.5%
not sugar-fed	32	9	23	
B				
sugar-fed	51	31	20	56.4%
not sugar-fed	27	14	13	

A = Refer to Table 64.

B = Refer to Table 64.

TABLE 71

Classification of the U.K. honey samples representing five pollens into 'sugar-fed' and 'not sugar-fed' groups by the SPSS

Sugar groups	No. of samples	SPSS predicted sugar group membership		Percent of group correctly classified by SPSS
		sugar-fed	not sugar-fed	
Brassica				
A				
sugar-fed	12	11	1	94.1%
not sugar-fed	5	0	5	
B				
sugar-fed	13	11	2	68.2%
not sugar-fed	9	5	4	
Trifolium repens				
A				
sugar-fed	7	7	0	91.7%
not sugar-fed	5	1	4	
B				
sugar-fed	8	8	0	66.7%
not sugar-fed	7	5	2	
Castanea				
A				
sugar-fed	3	3	0	100.0%
not sugar-fed	2	0	2	
B				
sugar-fed	10	8	2	75.0%
not sugar-fed	2	1	1	
Calluna				
A				
sugar-fed	1	1	0	100.0%
not sugar-fed	2	0	2	
B				
sugar-fed	1	1	0	100.0%
not sugar-fed	0	0	0	
Myosotis				
A				
Sugar-fed	3	3	0	100.0%
not sugar-fed	1	0	1	
B				
sugar-fed	3	2	1	50.0%
not sugar-fed	1	1	0	

A = Refer to Table 64.

B = Refer to Table 64.

glutamic acid, alanine, leucine and a constant derived by the SPSS; in the *Trifolium repens* group, the amino acids used were leucine, tyrosine and a constant derived by the SPSS; in the *Castanea* group, the amino acids used were aspartic acid, serine, alanine and a constant derived by the SPSS; in the *Calluna* group, the amino acid used was glutamic acid and a constant derived by the SPSS and in the *Myosotis* group, the amino acids used were serine, valine and a constant derived by the SPSS.

The results of feeding sugar to the honeybees in the winter and in the spring months were assessed in the following manner. The autumn fed samples were allocated into the 'not sugar-fed' group and the statistical analysis performed in the usual way. The predicted classification of the one hundred and seventy one honey samples selected by the SPSS have been given in Table 72. The Fisher's linear discriminant functions were calculated for the following amino acids. These were: lysine, serine, glycine, tyrosine, phenylalanine, and a constant derived by the SPSS.

As before the SPSS was specified to select samples into the 'sugar-red' and 'not sugar-fed' groups representing the five pollen. The predicted classification of the ninety honey samples selected by the SPSS have been given in Table 73.

The amino acids for which the Fisher's linear discriminant functions were calculated differed in four pollen groups. These differences were: in the *Brassica* group, the amino acids used were lysine, aspartic acid, threonine, glycine, alanine, isoleucine, leucine and a constant derived by the SPSS; in the *Trifolium repens* group, the amino acids used were lysine, isoleucine and a constant derived by the SPSS; in the *Castanea* group, the amino acids used were proline, glycine, alanine, leucine,

TABLE 72

Classification of the U.K. honey samples which were Winter and Spring fed into 'sugar-fed' and 'not sugar-fed' groups by the SPSS

Sugar groups	No. of samples	SPSS predicted sugar group membership		Percent of group correctly classified by SPSS
		sugar-fed	not sugar-fed	
A				
sugar-fed	15	9	6	69.5%
not sugar-fed	67	19	48	
B				
sugar-fed	24	15	9	57.3%
not sugar-fed	65	29	36	

A = Refer to Table 64.

B = Refer to Table 64.

TABLE 73

Classification of the U.K. honey samples representing four pollens into 'sugar-fed' and 'not sugar-fed' groups by the SPSS

Sugar groups	No. of samples	SPSS predicted sugar group membership		Percent of group correctly classified by SPSS
		sugar-fed	not sugar-fed	
Brassica				
A				
sugar-fed	6	6	0	91.3%
not sugar-fed	17	2	15	
B				
sugar-fed	3	1	2	31.3%
not sugar-fed	13	9	4	
Trifolium repens				
A				
sugar-fed	2	1	1	80.0%
not sugar-fed	8	1	7	
B				
sugar-fed	3	1	2	64.7%
not sugar-fed	14	4	10	
Castanea				
A				
sugar-fed	4	4	0	100.0%
not sugar-fed	3	0	3	
B				
sugar-fed	2	1	1	55.6%
not sugar-fed	7	3	4	
Myosotis				
A				
sugar-fed	1	1	0	100.0%
not sugar-fed	2	0	2	
B				
sugar-fed	1	1	0	60.0%
not sugar-fed	4	2	2	

A = Refer to Table 64

B = Refer to Table 64.

phenylalanine, and a constant derived by the SPSS and in the *Myosotis* group, the amino acid used was tyrosine and a constant derived by the SPSS.

The effects of sugar feeding on the relative concentration of the imino acid proline to those of the other individual twelve amino acids was evaluated. The autumn, winter and spring fed samples were used. The SPSS control file given in Appendix VI (c) was modified by using the control statements listed in Appendix VI(eii). The predicted classification of the one hundred and seventy three honey samples selected by the SPSS have been given in Table 74. The following ratios of the amino acids for which the Fisher's linear discriminant functions were calculated. These were: glycine/proline, isoleucine/proline and a constant derived by the SPSS.

Computer placements of honey samples which were both heated and sugar-fed or visa versa

The SPSS predicted classification for all the four combinations of the 'heated and sugar-fed' and the 'not heated and not sugar-fed' groups have been given in Tables 75, 77, 79 and 81.

In the 'heated and sugar-fed' and the 'not heated and not sugar-fed' combinations the SPSS selected seventy nine honey samples for analysis. The Fisher's linear discriminant functions were calculated for the following amino acids which were entered into the analysis. These were: lysine, serine, glycine, alanine, valine, isoleucine, leucine and a constant derived by the SPSS.

The SPSS selected ninety two honey samples for the 'heated and not sugar-fed' and the 'not heated and sugar-fed' combination. The

TABLE 74

Classification of the U.K. honey samples into 'sugar-fed' and 'not sugar-fed' groups by the SPSS

Sugar groups	No. of samples	SPSS predicted sugar group membership		Percent of group correctly classified by SPSS
		sugar-fed	not sugar-fed	
A				
sugar-fed	24	11	13	58.8%
not sugar-fed	56	20	36	
B				
sugar-fed	37	14	23	54.8%
not sugar-fed	56	19	37	

A = Refer to Table 64.

B = Refer to Table 64.

TABLE 75

Classification of the U.K. honey samples into 'heated and sugar-fed' and 'not-heated and not sugar-fed' groups by the SPSS

Heat and sugar groups	No. of samples	SPSS predicted heat and sugar group membership		Percent of group correctly classified by SPSS
		HT+SF	NHT+NSF	
A				
HT+SF	14	11	3	79.0%
NHT+NSF	24	5	19	
B				
HT+SF	12	5	7	61.0%
NHT+NSF	29	9	20	

TABLE 76

Classification of the *Brassica* honey samples into 'heated and sugar-fed' and 'not-heated and not sugar-fed' groups by the SPSS

Heat and sugar groups	No. of samples	SPSS predicted heat and sugar group membership		Percent of group correctly classified by SPSS
		HT+ST	NHT+NSF	
Brassica				
A				
HT+SF	8	7	1	86.7%
NHT+NSF	7	1	6	
B				
HT+SF	0	0	0	80.0%
NHT+NSF	5	1	4	

These notes are common to Tables 75 and 76.

A = Refer to Table 64.

B = Refer to Table 64.

HT = Refer to Table 75.

NHT = Refer to Table 75.

SF = Refer to Table 75.

NSF = Refer to Table 75.

\+ = Refer to Table 75.

TABLE 77

Classification of the U.K. honey samples into 'heated and not sugar-fed' and not heated and sugar-fed' groups by the SPSS

Heat and sugar group	No. of samples	SPSS predicted heat and sugar group membership		Percent of group correctly classified by SPSS
		HT+SF	NHT+NSF	
A				
HT+NSF	3	2	1	78.3%
NHT+SF	43	9	34	
B				
HT+NSF	3	2	1	82.6%
NHT+SF	43	7	36	

TABLE 78

Classification of the *Brassica* and *Trifolium repens* honey samples into 'heated and not sugar-fed' and 'not heated and sugar-fed' groups by the SPSS

Heat and sugar group	No. of samples	SPSS predicted heat and sugar group membership		Percent of group correctly classified by SPSS
		HT+SF	NHT+NSF	
Brassica				
A				
HT+NSF	1	1	0	100.0%
NHT+SF	7	0	7	
B				
HT+NSF	1	0	1	81.8%
NHT+SF	10	1	9	
Trifolium repens				
A				
HT+NSF	1	1	0	100.0%
NHT+SF	7	0	7	
B				
HT+NSF	0	0	0	100.0%
NHT+SF	7	0	7	

These notes are common to both Tables 77 and 78.

A = Refer to Table 64.

B = Refer to Table 64.

HT = Refer to Table 75.

NHT = Refer to Table 75.

SF = Refer to Table 75.

NSF = Refer to Table 75.

\+ = Refer to Table 75.

TABLE 79

Classification of the U.K. honey samples into 'heated and not sugar-fed' and 'not heated and not sugar-fed' groups by the SPSS

Heat and sugar group	No. of samples	SPSS predicted heat and sugar group membership		Percent of group correctly classified by SPSS
		HT+NHF	NHT+NSF	
A				
HT+NSF	3	2	1	97.0%
NHT+NSF	30	0	30	
B				
HT+NSF	3	1	2	76.9%
NHT+NSF	23	4	19	

TABLE 80

Classification of the *Brassica* honey samples into 'heated and not sugar-fed' and 'not heated and not sugar-fed' groups by the SPSS

Heat and sugar group	No. of samples	SPSS predicted heat and sugar group membership		Percent of group correctly classified by SPSS
		HT+NSF	NHT+NSF	
Brassica				
A				
HT+NSF	2	2	0	100.0%
NHT+NSF	4	0	4	
B				
HT+NSF	0	0	0	62.5%
NHT+NSF	8	3	5	

These notes are common to both Tables 79 and 80.

A = Refer to Table 64.

B = Refer to Table 64.

HT = Refer to Table 75.

NHT = Refer to Table 75.

NSF = Refer to Table 75.

\+ = Refer to Table 75.

Fisher's linear discriminant functions were calculated for the amino acid glutamic acid and a constant derived by the SPSS.

For the combination of 'heated and not sugar-fed' and 'not heated and not sugar-red', the SPSS selected fifty nine honey samples. The following amino acids were used for calculating the Fisher's linear discriminant functions. These were: aspartic acid, serine, glutamic acid, proline, tyrosine, phenylalanine and a constant derived by the SPSS.

In the final combination of the four, that is, the 'heated and sugar-fed' and 'not heated and sugar-red', the SPSS selected one hundred and twelve honey samples. The Fisher's linear discriminant functions were calculated for the following amino acids. These were: lysine, serine, glycine, valine, isoleucine, phenylalanine and a constant derived by the SPSS.

For each of the four combinations the SPSS was specified to select honey samples representing the five pollen groups.

In the first of the four combinations listed above the predicted classification was obtained for the *Brassica* group only. The results have been given in Table 76. In this group the SPSS selected twenty honey samples and the Fisher's linear discriminant functions were calculated for the following amino acids. These were: lysine, aspartic acid, phenylalanine and a constant derived by the SPSS.

In the second combination, the predicted classification were obtained for two pollen groups and these were: the *Brassica* and *Trifolium repens*. The results have been given in Table 78. In the *Brassica* group, the SPSS selected nineteen honey samples and the Fisher's linear discriminant functions were calculated for the following amino acids. These were:

aspartic acid, threonine, proline, valine, leucine, tyrosine and a constant derived by the SPSS. In the *Trifolium repens* group, the SPSS selected fifteen honey samples and the Fisher's linear discriminant functions were calculated for the following amino acids. These were: threonine, alanine, tyrosine, phenylalanine and a constant derived by the SPSS.

In the third combination, the predicted classification was obtained for *Brassica* group only. The results have been given in Table 80. The SPSS selected fourteen honey samples and the Fisher's linear discriminant functions were calculated for the following amino acids. These were: aspartic acid, glycine, isoleucine, phenylalanine and a constant derived by the SPSS.

In the fourth of the four combinations there were only two pollen groups for which the predicted classification were obtained. These were: the *Brassica* and *Castanea*. The results for these two pollen groups have been given in Table 82. In the *Brassica* group, the SPSS selected twenty five honey samples and the Fisher's linear discriminant functions were calculated for the following amino acids. These were: alanine, tyrosine and a constant derived by the SPSS. In the *Castanea* group, thirteen honey samples were selected by the SPSS and the Fisher's linear discriminant functions were calculated for the following amino acids. These were: aspartic acid, serine, proline, leucine, tyrosine and a constant derived by the SPSS.

TABLE 81

Classification of the U.K. honey samples into 'heated and sugar-fed' and 'not heated and sugar-fed' groups by the SPSS

Heat and sugar groups	No. of samples	SPSS predicted heat and sugar group membership		Percent of group correctly classified by SPSS
		HT+SF	NHT+SF	
A				
HT+SF	13	10	3	81.0%
NHT+SF	29	5	24	
B				
HT+SF	13	5	8	58.6%
NHT+SF	57	21	36	

TABLE 82

Classification of the *Brassica* and *Castanea* honey samples into 'heated and sugar-fed' and 'not heated and sugar-fed' groups by the SPSS

Heat and sugar groups	No. of samples	SPSS predicted heat and sugar group membership		Percent of groups correctly classified by SPSS
		HT+SF	NHT+SF	
Brassica				
A				
HT+SF	3	2	1	91.7%
NHT+SF	9	0	9	
B				
HT+SF	5	1	4	38.5%
NHT+SF	8	4	4	
Castanea				
A				
HT+SF	3	3	0	100.0%
NHT+SF	4	0	4	
B				
HT+SF	0	0	0	33.3%
NHT+SF	6	4	2	

These notes are common to both Tables 81 and 82.

A = Refer to Table 64.

B = Refer to Table 64.

HT = Refer to Table 75.

NHT = Refer to Table 75.

SF = Refer to Table 75.

\+ = Refer to Table 75.

Analyses of Samples from the Foreign and Commercial Honeys

Computer placement of honey samples into country groups

Discriminant analysis similar to that of the U.K. survey was performed on the modified amino acid concentration measurements of the sixty four foreign and commercial honey samples using the 'MAHAL' method. The SPSS allocated honey samples to the appropriate country group according to the criteria specified in the control statements 54 to 60 listed in Appendix VII(a). The predicted classification of the twenty three honey samples selected by the SPSS into the Australia, Canada, Mexico, New Zealand and English groups have been given in Table 83. The honey samples in each of the five country groups were divided into two subsets by the SPSS.

In one subset, the Fisher's linear discriminant functions were calculated for the following amino acids which were entered into the analysis. These were: lysine, serine, glutamic acid, glycine, valine, leucine, tyrosine and a constant derived by the SPSS. The twelve honey samples selected by the SPSS were 100.0% correctly classified into their appropriate country groups.

In the other subset, eleven honey samples were selected for estimating the misclassification rate and of these only 45.5% were correctly classified by the SPSS into their respective country groups.

There were six samples (Code Nos. 194, 195, 203, 204, 229 and 249) misclassified by the SPSS and these were experimentally shown to come from countries Australia, Canada, England, Mexico and New Zealand; the computer predictions suggested that five samples excluding 204 originated from Australia, Mexico and New Zealand. This is a reasonable predictive mistake because the pollen groups from families Cruciferae and Leguminosae

are in fact common to all these countries. The omitted sample 204 was experimentally shown to have no detectable pollen present.

The following predicted classification was obtained by the SPSS when the English commercial samples were omitted from the previous analysis. The results have been shown in Table 84. The nine honey samples selected for Fisher's linear discriminant function calculation were 100.0% correctly classified. The following amino acids were entered into the analysis. These were: lysine, aspartic acid, glutamic acid, proline, alanine, phenylalanine and a constant derived by the SPSS. There were seven samples selected by the SPSS for estimating the misclassiciation rate and only 42.9% of the samples were correctly classified.

There were four samples (Code Nos. 195, 202, 227 and 228) misclassified by the SPSS. It should be pointed out that the pollen composition of 195 and 202 were similar to that expected from their predicted countries namely Canada and New Zealand, respectively. The computer predicted 227 was from Canada and 228 from New Zealand and pollen patterns were not dissimilar to those expected if the predicted origins were correct.

When the China honey samples were included in the above analysis the predicted classifications shown in Table 85 were obtained by the SPSS. The nine honey samples selected for calculating the Fisher's linear discriminant functions were 100.0% correctly classified. The following amino acids were entered into the analysis and these were: glutamic acid, alanine, valine, tyrosine, phenylalanine and a constant derived by the SPSS. There were thirteen honey samples selected for estimating the misclassification rate and of these only 15.4% were correctly classified.

TABLE 83

Classification of the foreign and commercial honey samples into five country groups by the SPSS

Country groups	No. of samples	SPSS predicted country group membership				
		Australia	Canada	Mexico	New Zealand	English
A						
Australia	2	2	0	0	0	0
Canada	5	0	5	0	0	0
Mexico	2	0	0	2	0	0
New Zealand	1	0	0	0	1	0
English	2	0	0	0	0	2
B						
Australia	2	0	0	1	1	0
Canada	3	1	1	0	1	0
Mexico	0	0	0	0	0	0
New Zealand	1	1	0	0	0	0
English	5	1	0	0	0	4

TABLE 84

Classification of the foreign and commercial honey samples into four country groups by the SPSS

Country groups	No. of samples	SPSS predicted country group membership			
		Australia	Canada	Mexico	New Zealand
A					
Australia	3	3	0	0	0
Canada	4	0	4	0	0
Mexico	0	0	0	0	0
New Zealand	2	0	0	0	2
B					
Australia	1	0	1	0	0
Canada	4	0	3	0	1
Mexico	2	0	1	0	1
New Zealand	0	0	0	0	0

These notes are common to both Tables 83 and 84.

A = Refer to Table 64.

B = Refer to Table 64.

TABLE 85

Classification of the foreign and commercial honey samples into five country groups by the SPSS

Country groups	No. of samples	SPSS predicted country group membership				
		Australia	Canada	China	Mexico	New Zealand
[A]Australia	1	1	0	0	0	0
Canada	3	0	3	0	0	0
China	3	0	0	3	0	0
Mexico	0	0	0	0	0	0
New Zealand	2	0	0	0	0	2
[B]Australia	3	0	0	1	0	2
Canada	5	0	1	2	0	2
China	3	0	1	1	0	1
Mexico	2	2	0	0	0	0
New Zealand	0	0	0	0	0	0

A = Refer to Table 64.

B = Refer to Table 64.

There were eleven honey samples misclassified by the SPSS and of these four samples had pollen composition similar to the country they were predicted to have originated. These four samples were: Australia Code Nos. 195 and 196, Canada Code No. 201 and Mexico Code No. 228. The remaining seven honey samples had pollen composition dissimilar to that predicted by the SPSS. These seven samples were: Australia Code No. 197, Canada Code Nos. 200, 203 and 204, China Code Nos. 208 and 209 and Mexico Code No. 227.

The five honey samples from France were also included in the above discriminant analysis and the predicted classifications shown in Table 86 were obtained by the SPSS. The seven honey samples selected for calculating the Fisher's linear discriminant functions were 100.0% correctly classified. The following amino acids were entered into the analysis and these were: threonine, alanine, isoleucine and a constant derived by the SPSS. There were twenty honey samples selected for estimating the misclassification rate and of these only 35.0% were correctly classified.

There were twelve honey samples misclassified by the SPSS and of these six samples had pollen composition similar to the country they were predicted to have originated. These six samples were: Australia Code No. 196, Canada Code No. 202, France Code Nos. 215, 216, 217 and 218. The remaining six samples had pollen composition dissimilar to that predicted by the SPSS. These six samples were: Australia Code Nos. 194, 195 and 197 and China Code Nos. 208, 211 and 213.

In the above analysis, the honey samples of Mexico and New Zealand were replaced by those from India and the predicted classifications

TABLE 86

Classification of the foreign and commercial honey samples into six country groups by the SPSS

Country group	No. of samples	SPSS predicted country group membership					
		Australia	Canada	China	France	Mexico	New Zealand
[A] Australia	0	0	0	0	0	0	0
Canada	2	0	2	0	0	0	0
China	2	0	0	2	0	0	0
France	0	0	0	0	0	0	0
Mexico	1	0	0	0	0	1	0
New Zealand	2	0	0	0	0	0	2
[B] Australia	4	0	2	1	0	0	1
Canada	6	0	5	0	0	0	1
China	4	0	1	1	0	1	1
France	5	0	3	0	0	1	1
Mexico	1	0	0	0	0	1	0
New Zealand	0	0	0	0	0	0	0

A = Refer to Table 64.

B = Refer to Table 64.

obtained by the SPSS have been shown in Table 87. The eleven honey samples selected for calculation of the Fisher's linear discriminant functions were 100.0% correctly classified. The following amino acids were entered into the analysis and these were: glutamic acid, proline, glycine, isoleucine, leucine, phenylalanine and a constant derived by the SPSS. There were fifteen honey samples selected for estimating the misclassification rate and of these 40.0% were correctly classified.

There were nine honey samples misclassified by the SPSS and of these five had pollen composition similar to the country they were predicted to have originated. These five samples were: Canada Code Nos. 200 and 206, China Code No. 208, France Code Nos. 215 and 216. The remaining four samples had pollen composition dissimilar to that predicted by the SPSS. These four samples were: Australia Code No. 197, Canada Code Nos. 202 and 203 and China Code No. 209.

Discriminant analysis was carried out between the English and the European sample. The European samples included the honeys from France and Spain. The predicted classifications obtained by the SPSS have been shown in Table 88. The seven samples selected for calculation of the Fisher's linear discriminant functions were 100.0% correctly classified. The following amino acids were entered into the analysis. These were: threonine, glutamic acid, valine, phenylalanine and a constant derived by the SPSS. There were seven samples selected for estimating the misclassification rate and of these only 71.4% were correctly classified.

There were two samples (Code Nos. 216 and 232) which were misclassified by the SPSS, however their pollen composition was similar to that expected from their predicted origins.

TABLE 87

Classification of the foreign and commercial honey samples into five country groups by the SPSS

Country groups	No. of samples	SPSS predicted country group membership				
		Australia	Canada	China	France	India
[A]Australia	2	2	0	0	0	0
Canada	2	0	2	0	0	0
China	2	0	0	2	0	0
France	2	0	0	0	2	0
India	3	0	0	0	0	3
[B]Australia	2	1	0	1	0	0
Canada	6	0	2	0	3	1
China	4	0	1	2	0	1
France	3	0	2	0	1	0
India	0	0	0	0	0	0

A = Refer to Table 64.

B = Refer to Table 64.

Computer placements of honey samples into the honey source groups

The SPSS was specified to recode and allocate honey samples according to their appropriate honey source groups by replacing the control statements given in Appendix VII(a) by those given in Appendix VII(bvi) as described. The predicted classification obtained by the SPSS have been shown in Table 89. The ten honey samples selected and then divided into the two subsets were 100.0% correctly classified. The Fisher's linear discriminant functions were calculated for the following amino acids which were entered into the analysis. The amino acids were: glutamic acid, phenylalanine and a constant derived by the SPSS.

Computer placements of honey samples into pollen groups

The control statements given in Appendix VII(bvii) were used in the following analysis by replacing those given in Appendix VII(a) as described. The predicted classification of the honey samples into the *Trifolium repens* and *Melilotus* groups by the SPSS have been shown in Table 90. The three honey samples selected for calculation of the Fisher's linear discriminant functions were 100.0% correctly classified. The amino acid serine and a constant derived by the SPSS were entered into the analysis. There were three honey samples selected for estimating the misclassification rate and of these only 66.7% were correctly classified.

The misclassified New Zealand sample Code No. 230 did not contain the pollen predicted by the SPSS.

Including the honey samples containing the *Lotus* pollen in the above analysis, that is, discriminating between pollen groups originating from the Leguminosae plant family. The predicted classification obtained by the SPSS have been shown in Table 91. The three samples selected

TABLE 88

Classification of the English and European honey samples by the SPSS

Country groups	No. of samples	SPSS predicted country group membership		
		France	Spain	English
A				
France	3	3	0	0
Spain	1	0	1	0
English	3	0	0	3
B				
France	2	1	1	0
Spain	1	0	0	1
English	4	0	0	4

TABLE 89

Classification of the floral honey samples by the SPSS

Floral groups	No. of samples	SPSS predicted floral group membership	
		Honeydew	Nectar
A			
Honeydew	2	2	0
Nectar	2	0	2
B Honeydew	1	1	0
Nectar	5	0	5

TABLE 90

Classification of the *Trifolium repens* and *Melilotus* honey samples by the SPSS

Pollen groups	No. of samples	SPSS predicted pollen group membership	
		308*	337*
A			
308*	2	2	0
337*	1	0	1
B			
308*	2	1	1
337*	1	0	1

TABLE 91

Classification of the honey samples belonging to the Papilionaceae plant family by the SPSS

Pollen groups	No. of samples	SPSS predicted pollen group membership		
		325*	337*	308*
A				
325*	1	1	0	0
337*	0	0	0	0
308*	2	0	0	2
B				
325*	1	0	0	1
337*	2	1	0	1
308*	2	0	0	2

These notes are common to Tables 88, 89, 90 and 91.

A = Refer to Table 64.

B = Refer to Table 64.

* = Refer to Table 30.

for Fisher's linear discriminant function calculations were 100.0% correctly classified. The amino acid glycine and a constant derived by the SPSS were entered into the analysis. There were five honey samples selected for estimating the misclassiciation rate and of these only 40.0% were correctly classified.

There were three honey samples Code Nos. 203, 209 and 226 which were misclassified and these did not contain the predicted pollen.

Computer placements of honey samples of unknown origin into country groups

The appropriate country code number was allocated to honey samples of unknown country source by evaluation of their pollen content and amino acid maps. The SPSS control file detailed in Appendix VII(a) was used and the predicted classifications obtained have been shown in Table 92. The eighteen honey samples selected for calculating Fisher's linear discriminant functions were 100.0% correctly classified. The following amino acids were entered into the anlysis and these were: aspartic acid, threonine, glutamic acid, proline, glycine, alanine, leucine, phenylalanine and a constant derived by the SPSS. There were thirteen samples selected for estimating the misclassiciation rate and of these only 46.2% were correctly classified.

There were seven honey samples misclassified by the SPSS and of these five had pollen composition similar to that predicted by the SPSS. These five samples were: Australia Code No. 196, Canada Code Nos. 200 and 205, New Zealand Code No. 230 and Russia Code No. 231. In the remaining two honey samples, the pollen composition of Canada Code No. 204 was not known and the pollen composition of Yellow Box sample Code

No. 246 was not similar to that of the Canada honeys.

Computer placements of honey samples that were commercially processed or otherwise

The control statements 54 to 58 of the SPSS control file given in Appendix VII(a) were modified as described. The predicted classifications obtained by the SPSS have been shown in Table 93. The twenty honey samples selected for calculation of Fisher's linear discriminant functions were 80.0% correctly classified. The following amino acids were entered into the anlaysis and these were: threonine, glycine and a constant derived by the SPSS. There were thirty four honey samples selected for estimating the misclassification rate and of these only 64.7% were correctly classified.

No distinct pattern became evident in the samples that were misclassified by the SPSS.

Evaluation of Analytical Data of Honey Samples From All Sources

Computer placements of honey samples into the United Kingdom and Foreign and commercial groups

The U.K. survey and the foreign and commercial data files were combined and the SPSS control file detailed in Appendix VI(c) was modified as described previously. The predicted classification of honey samples by the SPSS have been shown in Table 94. The combined honey samples of the two groups were divided into two subsets by the SPSS. In one subset, one hundred and twenty five honey samples were selected for calculation of Fisher's linear discriminant functions and these were 79.2% correctly classified. The following amino acids were entered into

TABLE 92

Classification of the unknown country of origin honey samples into country groups by the SPSS

Country groups	No. of samples	SPSS predicted country group membership			
		Australia	Canada	Mexico	New Zealand
A					
Australia	7	7	0	0	0
Canada	4	0	4	0	0
Mexico	5	0	0	5	0
New Zealand	2	0	0	0	2
B					
Australia	6	4	2	0	0
Canada	3	2	1	0	0
Mexico	3	1	1	1	0
New Zealand	1	1	0	0	0

TABLE 93

Classification of the foreign and commercial honey samples into 'processed' and 'unprocessed' groups by the SPSS

Process groups	No. of samples	SPSS predicted process group membership	
		Processed	Unprocessed
A			
Processed	11	10	1
Unprocessed	9	3	6
B			
Processed	19	15	4
Unprocessed	15	8	7

These notes are common to Tables 92 and 93.

A = Refer to Table 64.

B = Refer to Table 64.

the analysis and these were: lysine, threonine, glutamic acid, glycine, alanine, valine, tyrosine, phenylalanine and a constant derived by the SPSS. There were one hundred and thirty one honey samples selected for estimating the misclassification rate and of these only 72.5% were correctly classified.

No distinct pattern became evident in the misclassified honey samples of U.K. survey and foreign and commercial groups which could explain the results.

Computer placements of honey samples into the country groups

The SPSS was specified to select honey samples into the United Kingdom, Australia, Canada, China, France, India and English groups as detailed in the control statements listed in Appendix VII(cii). The predicted classifications obtained by the SPSS have been given in Table 95. The one hundred and fourteen honey samples selected for calculation of Fisher's linear discriminant function only 74.6% of the samples were correctly classified into their respective country groups. The following amino acids were entered into the analysis and these were lysine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine and a constant derived by the SPSS. There were one hundred and ten honey samples selected for estimating the misclassification rate and of these only 57.3% were correctly classified.

The predicted pollen composition of all the misclassified samples were similar to the allocated country groups, by the SPSS.

Computer placements of honey samples into pollen groups

The SPSS was specified to allocate honey samples into the *Brassica*, *Trifolium repens* and *Castanea* pollen groups as described. The SPSS selected forty seven honey samples out of the two hundred and fifty six samples to perform discriminant analysis. The predicted classifications obtained by the SPSS have been shown in Table 96. The twenty two samples selected for calculating Fisher's linear discriminant functions were 100.0% correctly classified. The following amino acids were entered into the analysis and these were: lysine, threonine, glutamic acid, alanine, isoleucine, leucine, tyrosine, phenylalanine and a constant derived by the SPSS. There were twenty five honey samples selected for estimating the misclassification rate and of these only 68.0% were correctly classified.

There were seven honey samples misclassified by the SPSS and the presence of *Trifolium repens* pollen was correctly predicted to be present in the U.K. sample Code No. 178. The remaining six samples, U.K. survey Code Nos. 26, 39, 64, 68, 102 and the Canada Code No. 202 the pollen composition predicted did not agree with that present in these samples.

TABLE 94

Classification of the combined honey samples into the U.K. and foreign and commercial groups by the SPSS

Country groups	No. of samples	SPSS predicted country group membership	
		U.K.	F.C.
A			
U.K.	97	83	14
F.C.	28	12	16
B			
U.K.	96	74	22
F.C.	35	14	21

TABLE 95

Classification of the combined honey samples into seven country groups by the SPSS

Country groups	No. of samples	SPSS predicted country group membership						
		U.K.	Australia	Canada	China	France	India	English
A								
U.K.	99	71	1	14	0	6	0	7
Australia	3	0	3	0	0	0	0	0
Canada	1	0	0	1	0	0	0	0
China	3	0	0	0	3	0	0	0
France	3	0	0	0	0	3	0	0
India	2	0	0	0	0	0	2	0
English	3	1	0	0	0	0	0	2
B								
U.K.	92	59	3	12	1	8	2	7
Australia	1	0	1	0	0	0	0	0
Canada	7	6	0	0	0	1	0	0
China	3	1	0	0	1	1	0	0
France	2	1	0	0	0	1	0	0
India	1	0	0	0	0	0	1	0
Enlish	4	3	0	0	0	1	0	0

TABLE 96

Classification of the combined honey samples into three pollen groups by the SPSS

Pollen groups	No. of samples	SPSS predicted pollen group membership		
		301*	308*	318*
A				
301*	15	15	0	0
308*	2	0	2	0
318*	5	0	0	5
B				
301*	18	4	0	4
308*	3	2	1	0
318*	4	1	1	2

These notes are common to Tables 94, 95 and 96.

A = Refer to Table 64.

B = Refer to Table 64.

U.K. = United Kingdom

F.C. = Foreign and commercial

* = Refer to Table 30.

D I S C U S S I O N

CHAPTER 4

D I S C U S S I O N

CHAPTER 4

PRELIMINARY SUBJECTIVE EXAMINATION OF HONEY SAMPLES

The quality of honey is generally assessed on the basis of its colour and flavour. The light colour class honeys are usually associated with a mild flavour while the dark colour class honeys with strong flavours. The colour and flavour of a honey are different to the plant from which the nectar was originally gathered by the honeybee. However, these two properties of honey are greatly influenced by a number of factors. These are beekeeping, handling, processing and storage. It has been generally reported by various researchers that heating and storage of honey under unfavourable conditions results in an increase in the darkening, loss of flavour and an increase in its hydroxymethylfurfural content. These researchers were: White, Kushnir and Subers (1964); Echigo, Takenaka and Ichimura (1974) and White (1978). Blending of honeys also effects its colour. It was reported by Milium (1939) that combination of tannates and polyphenols with iron from containers and processing equipment results in colour formation during storage. The formation of formaldehyde derivatives and changes due to heating and caramelisation are contributing factors. Also, factors such as the non-enzymic browning reactions involving the Maillard and other reactions which usually lead to the production of reactive intermediates such as Schiff's bases and subsequent further reactions should also be considered. The enzyme browning reaction using the enzymes and phenols derived from the original nectar would also be expected to contribute in unheated honey samples. The absence of significant amounts of the

aromatic amino acids such as tryptophan and tyrosine in light colour class honeys and their presence in dark colour class honeys were noted by Phadke (1962). It was reported by Browne (1908) that dark coloured honeys gave intense colour reactions when tested for polyphenolic compounds. Further, some of the constituents named above such as various carbohydrates, amino and other acids, tanins and minor non-volatiles contributes towards the aroma and flavours of honey. Other components such as alkaloids, aromatic compounds, aldehydes, esters, alcohols, phenylethyl and benzyl alcohols have been reported present in honey by Ten-Hoopen (1963) and Cremer and Riedmann (1965). Most of these compounds are the resultant of the process of degradation or reactions of amino acids with other constituents of honey. Compounds such as methyl anthranilate have been isolated from orange and lavender honey which gives these honeys their characteristic aroma. Other factors could also influence the flavour and colour of honey. These factors could be introduced into the honey from the gathering of nectar by the honeybee and during the ripening, extraction of honey and the manipulations to achieve the final bottled product. These factors could be compounds such as pesticides; honeybee repellants such as smoke, benzylaldehyde or sulphur dioxide; burnt wax resultant of excessive heating which also causes an increase in hydroxymethylfurfural content; fermentation occuring from addition of water or blending with high yeast content honeys, honeydew or foreign honeys and even lubricating oil from machinery during processing.

In the present study most of the foreign honeys, especially the Mexican, had very high phenylalanine content followed by Australia, China, Yellow Box and samples of Gale honey. Also, some of the

U.K. survey honey had high phenylalanine content. These were: sample nos. 53, 158, 172, 173, 174, 183, 184 and 191. This phenylalanine high content could be a result of protein breakdown due to excessive heating and storage in unfavourable conditions, especially the foreign honeys. The depression of the other amino acids could be the result of condensation reaction with other honey constituents especially the sugars. It is suggested that these high phenylalanine honeys should not be consumed by persons who suffer from the genetic disorder of phenylketonuria which affects the phenylalanine metabolism.

ASSESSMENT OF PRESENT WORK

Proteins

Isolation

Due to the very viscous nature of honey a given sample of honey had to be diluted (1:2) with distilled water before any attempt could be made to concentrate and then isolate the proteins present. Several standard techniques for separating proteins were considered for use on honey and these were: dialysis, gel filtration (sephadex), ion-exchange chromatography and ultrafiltration. The advantages and disadvantages of application of each of these methods were evaluated for honey.

Dialysis

The advantages of the dialysis procedure are that it is a gentle process which can, if desired, be conducted at a low temperature and usually results in a 2 - 3 fold increase of the protein solution. However, with this method the disadvantages outweigh the advantages

in that the procedure requires the use of large volumes of solutions and thus, another separate method is needed to reduce the final volume. The complete procedure of dialysis takes a long time in order of days, four to five, to separate proteins from dialysable smaller molecular weight material and then the protein solution has to be concentrated as a separate step. This length of experimental time also presents risks due to microbial contamination of the dialysis tubing and the diluted honey solutions.

Gel filtration

The technique of gel filtration using sephadex as medium is considered to be an efficient technique and would result in the separation of proteins from carbohydrates. The advantage of this method over dialysis is that it enables desalting as well as protein concentration. However, the disadvantages of using sephadex gel filtration on honey are that this technique ideally requires small amounts of pure protein mixtures containing a number of different sized molecules eluting at different positions. Due to the presence of carbohydrates the dilution of honey was a must and therefore, large quantities of solutions are needed for filtration. This can be overcome by performing the technique of gel filtration under pressure but would result in the compression of the gel bed. Further, this method only separates according to molecular size and presence of polysaccharides would create problems such as distortion of protein elution. Furthermore, the sephadex is liable to irreversible absorption of small amounts of proteins and losses increased as the amount of protein approached milligram quantities would lead to significant errors in the final calculations of recoveries (Washington, 1966).

Ion-exchange chromatography

Due to the viscosity of honey a large mesh size resin would be required and the technique of ion-exchange chromatography was found to be advantageous in the separation of amino acids and peptides. Thorough washing after binding results in the loss of all the carbohydrates and proteins thus, enabling the elution of the amino acids using 7M ammonium hydroxide solution. If proteins only were required to be eluted than inevitably amino acids would be present in the final extract. Therefore, the total isolation of proteins by ion-exchange would be difficult. Another reason for this difficulty would be the poor absorption onto the ion-exchange resin partly due to sizes of the protein molecules and also because proteins are weakly charged molecules. Moreover, the stability of the protein molecules in the separating media whether be it acidic or alkaline would result in the elution of proteins just after the polysaccharides and carbohydrates. This would lead to trailing throughout the column. Furthermore, if separation of proteins is to be efficient a loosely cross-linked resin of x2 or x4% divinylbenzene is needed rather than the more rigid x8% cross-link of resin routinely used for amino acids. Also due to the restricted applications of these loosely cross-linked resins they are often only available for high resolution techniques utilizing chromatographic grade resin of approximately 400 mesh. This factor would preclude use for honey as done in the present study. It was reported by Schepartz and Subers (1964) that some of the honey enzymes such as glucose oxidase were binding irreversibly or not binding at all with ion-exchange media such as DEAE- and CM-cellulose, respectively. Therefore, losses similar to that encountered with basic

amino acids would also result here.

Ultrafiltration

For the reasons given above the technique of ultrafiltration was selected for concentrating the proteins present in honey. The advantages of this technique are that the volumes of protein solutions remain approximately unchanged and the process seems to be reasonably quick for normal protein solutions at room temperature. The main reason for choosing this technique was that it enabled the selection of a filter membrane with a specific pore size so that molecules above a specific molecular size can be retained. Assuming that the proteins used for initial industrial calibration were similar to those of honey, but there is no evidence on this point.

However, during ultrafiltration it was observed that the through put of the honey solution in the amicon cell was much slower, resulting in three to four days for the removal of all the soluble filterable components. The reasons for this could be due to:

1. the presence of large inclusions in honey, even after clarification, could be blocking the pores of the UM10 filter membrane even though stirring was available to minimise this effect;
2. the fact that even after dilution the honey solution still possessed considerable viscosity when compared to water or dilute solutions.

It was also found that even though precautions were taken to perform the ultrafiltration procedure at a temperature of $4^{o}C$ discolouration due to microbial contamination of the inner walls of the amicon cells and the outlet tubing usually occurred after the third day of the

experiment. An odour similar to that of alcohol was evident when buffer solutions were added to the concentrated solution in the amicon cell.

The residue retained by the UM10 disc consists of those molecules whose average molecular size was above the 10,000 dalton cut off limit of the filter membrane. Depending on how the filter membrane was calibrated not all the material retained may have a molecular weight above 10,000, and molecular size and shape are equally important especially if polysaccharides are involved. After freeze-drying the residue above the membrane, it was necessary to store in a dessicator over phosphorous pentoxide to prevent access of moisture to this hygroscopic material. Even with precautions some of the protein concentrates were usually gummy and sticky perhaps due to traces of polysaccharides which may be present. It was because of this that the protein concentration was determined by the Lowry *et al.*, (1951) method within a week after freeze-drying.

Concentration Measurements

The method of Lowry *et al.*, (1951) for many years was extensively used to measure protein concentration in a sample relative to a standard, usually albumin. However, this method has several disadvantages including that of complicated labile reagents, unstable colour and more importantly proteins of different composition give a very different response than did the standard at equal concentration of proteins. For these reasons Bradford (1976) searched for a different technique free of these errors and was successful in developing a dye-protein method and this was taken up commercially as kits. Typical kits such as that

by 'Pierce' are one-step procedures, giving a stable colour and giving similar responses for most proteins, even at low concentrations. The method relies on a shift in absorbance of the acidic Coomassie Brilliant Blue G250 from free to protein bound state. This reagent is regarded as being extremely sensitive to low protein concentrations, unaffected by interferring substances and is stable for months.

Preliminary comparisons of the Lowry and dye-binding methods as applied to the honey protein concentrates showed that the latter method was atypically erratic with inconsistent absorbance valves on replicates. In contrast the Lowry reagent gave reasonable and reproducible absorbance measurements. On the basis of this the Lowry method was adopted for use on honey concentrates. The reason for the Pierce reagent being unsuitable for use on honey concentrates could be due to honey components such as gum, polysaccharides, residual wax, pollen or spores. One explanation could be that the acidic coomassie brilliant blue could be reacting with the pollen exine in a similar manner to that of basic fushin. The differences in amount and distribution of sporopollinin on the surface of pollen exine between different species could be responsible for the inconsistent absorbance measurements.

Polyacrylamide Gel Electrophoresis

Examination of honey concentrates by PAGE or SDS-PAGE gave protein bands (Figure 3) however, the variation in the position of the protein bands precluded using this technique for honey proteins as a means of detecting sample source chemically rather than by pollen estimation. Furthermore, very recently it was shown by Reeder, Richie and Guenther (1986) that it was possible to distinguish honey based on geographical

location of collection using state of the art technology. This advanced technology incorporated ultra-thin layer isoelectric focusing, laser scanner and a chemometric programme on a mainframe computer. Moreover, this form of technology may be available at nationally or more likely at internationally based research centres and the application of such technology to only a few honeys is not only uneconomical but would not justify such equipment because of the accumulated data to form a data bank. Therefore, it is considered economically necessary to either improve the precision of existing commonly used laboratory techniques such as PAGE or investigate other minor constituents of honey such as amino acids. This was one of the aims of the present study as pollens present can be partially or completely removed by pressure filtration in the commercial interest of presenting a crystal clear product of extended shelf-life and good appearance. In the absence of pollen conventional identification is impossible.

Heating of honey for liquification or other commercial treatment usually results in the coagulation and/or denaturation of the proteins present. This was evident in the protein band patterns observed on the 5% and 10% SDS-PAGE gels of the not heat treated and heat treated samples of English Chorley honey No. 261, see Figure 3. Further, the fact that the protein band pattern of the heat treated sample No. 261 and the commercial samples (fourteen) used in this analysis, refer to Tables 10 and 11, suggests that the normal processing method after extraction was effecting the nature of the honey proteins. Furthermore,

the effects of heating honey were observed when the China Buckwheat honey sample Code No. 209 of necessity was subjected to heating upon receiving in order to remove glucose monohydrate crystals and debri such as bee fragments and wax. The prepared sample was applied to the SDS-PAGE gels. The polyacrylamide gels loaded with China Buckwheat showed a very high background of protein even after sufficient dilution of the original honey applied. This suggests that the coagulated proteins were infact precipitating followed by dissolution of the precipitates during electrophoresis. When both the raw honey and honey concentrates were examined by applying to straight PAGE, the protein bands obtained could not be reproduced accurately. This lack of reproducibility suggests that the proteins present in the honey examined were either coagulated or proteolysis had occurred at some stage of sample preparation. However, the protein bands obtained on the 5% and 10% SDS-PAGE gels showed consistency in the patterns suggesting that the honeys examined were subjected to some degree of heating and excessive heating prior to being received in the laboratory as was the case with China buckwheat honey.

The scanning of the stained polyacrylamide gels was relatively simple. Tears in gels were usually recorded as peaks and were easily recognised. Gels scanned against a clear background, the peaks obtained due to the protein bands were usually sharp and well defined, refer to Figures 5 and 6.

Enzymes

For convenience proteins with catalytic powers namely enzymes are dealt with below. Enzymes and proteins are denatured in SDS-PAGE but the enzyme activity is retained on PAGE gels alone. This latter technique was used to particularly separate enzymes and other compounds. Their existence was subsequently sort by incubating the completed gels with the appropriate substrate. Although pilot studies with laboratory enzymes were successful, the definite existence of the following enzymes in honey, that is, diastase, glucose oxidase and invertase was not successful. The sort looked for detection of honey enzymes on PAGE gels may not have been successful for the following reasons:

1. The laboratory standard enzymes obtained from micro-organisms have different characteristic properties compared to those found in honey, although they catalyse the same substrate.
2. The raw honey applied (100 µl) although at the volume limit handled by these gels may not have sufficient enzyme or enzyme acitvity to indicate their presence by the technique employed.
3. Even if sufficient enzymes were present in the amount of honey applied, the enzymes could have leached out from their position on the gels during incubation.

This could explain why the presence of the standard laboratory glucose oxidase enzyme and its activity were not detected on the appropriate gels.

Furthermore, the U.K. survey samples were not subjected to PAGE after the completion of amino acid and pollen analyses for the following reasons:

1. The U.K. survey was scheduled from October 1981 through to October 1982 but even with pre-paid mailing service the samples came in very erratically and a substantial number were still coming in

even as late as 1984.

2. It was not possible to attempt correlation between variables until all the samples had been analysed and the final finite number of variables involved for correlation had been determined. The major variables considered were the quantitative individual concentration measurements for thirteen amino acids, the qualitative presence or absence of seventeen amino acids, heating of honey or otherwise, feeding of sugar to honeybees or otherwise, addition of sugar compounds such as syrups, floral sources as suggested by the beekeepers, or confirmed by pollen analysis, pollen type including the dangers of under- or over-representation as given by Sawyer (1975), commercial processing or otherwise and other information. This range of factors could not be handled by a simple micro-computer such as an Apple or BBC1, therefore, a Prime mainframe computer had to be used.

3. Furthermore, due to the large number of factors and consequent small numbers in each sub group special modifications to the SPSS package had to be made initially by the statistician, and subsequently further modifications to the treatment of data to prevent under-weighting of small sub groups involved.

4. The modifications that became necessary both initially and after the programme had been running for some time were such that they could not be dealt with within the simple operating programme that was being used. Therefore, I had to await the availability of the statistician who was experienced in the aspects of computer orientated statistics.

Ninhydrin Positive Substances

Covered under this section are the amino acids, amines including amino sugars, amides and small polypeptides. For reasons explained previously most of the present work was concerned with the free amino acids. It was because of this that the following procedures given in the sequence and order of stages were necessary so that analysis such as detection, identification and quantitation could be carried out. The sequence and order of the ion-exchange procedure were: protonation of the ion-exchange resin, binding of the amino acids to the resin, removal of carbohydrates, elution of the amino acids, evaporation under reduced pressure, desiccation under vacuum, re-constitution and then storage. Extracts were analysed by paper electrophoresis plus chromatography, automatic amino acid analyser, pollen analysis and computational analysis.

Ion-exchange Chromatography

The viscosity of honey necissitated the use of a large mesh cation ion-exchange resin for the isolation of ninhydrin positive substances. The feasibility of column chromatography or continually stirred batchwise operation of the large mesh resin was considered. The procedure of column chromatography was assessed to be too slow in view of the two hundred and fifty six samples. If column chromatography was used excessive dilution in the order of ten times or more was found to be necessary to be able to establish a reasonable flow rate. This also meant that large volumes of eluents would be required to be concentrated by evaporating under reduced pressure. Furthermore, the column chromatography technique was found to be prone to leakages and especially clogging of the tubing, nylon mesh filter and the resin column itself

by large particulates such as pollen, spores, soot, dust and other inclusions normally present in honey. This was considered to be a time consuming process with risk of loss of components. On the other hand the technique of batchwise extraction enabled controlled and sufficient dilution with stirring to enable effective binding of ninhydrin positive substances. Thus, the volume of eluents required for evaporation were about or less than one-fifth of those obtained for column chromatography. This is turn, also enabled accurate and quantitative determinations to be carried out.

The ion-exchange chromatography technique devised by Siddiqi (1981) was modified and improved to ensure optimum quantitative determination. The final method was assessed for the precision and reproducibility using solutions A2 and S1. These solutions contained known amounts of thirteen amino acids in the appropriate media. Further, solutions B and B1 contained amino acids plus sugars - glucose and fructose to provide a suitable standard for honey samples. The thirteen amino acids were: lysine, aspartic acid, serine, threonine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine and phenylalanine; and these solutions were deliberately standardised so as to represent the concentration of amino acids found in a typical honey. The loss of amino acids may occur at the following stages of the isolation procedure: preparation of honey samples, transfer to the resin, binding of the amino acids to the resin, washing to remove carbohydrates and other components, elution of amino acids from the resin, collection and evaporation under reduced pressure, desiccation, re-constitution, storage of prepared extract, operational loss for paper electrophoresis and chromatography and operational loss for the automatic amino acid analyser.

Due to the multiple stages involved and possible adverse effects of carbohydrates, it was essential to formulate recovery experiments which covered each stage separately as far as possible. These recovery experiments, Tables 13 to 29 show, with a few exceptions, that the recovery and total were satisfactory with recoveries greater than 98.8%. These aspects mentioned above will be reviewed separately.

Quantitative determination of amino acids

Once these standards were prepared by weighing, it was essential to verify that no gross errors had occurred and concentrations were as expected. For this reason these were submitted to automatic amino acid analysis which had been made ready and pre-calibrated with commercial standards as described. This solution A was shown to be satisfactory and gross errors due to dampness and incorrect weighing were not present, and recoveries of 101.3% were obtained within the guidelines of Moore and Stein (1951). Furthermore, the autoanalyser was satisfactory for the range and concentration of the amino acids expected in honeys. The standards in the various media and stages were likewise individually examined and were controls for each stage.

Due to the varying nature of the work load related to the numbers of samples received per week a rigid recovery schedule could not be operated. However, periodic checks were carried out on all aspects of the final procedure.

The final extract was found to be free of protein and therefore, these extracts were not furthered to a deproteinisation step. This step was not only unnecessary but had the danger of carrying down traces of amino acids, other ninhydrin positive substances and other components present in such extracts, with the precipitate.

Preparation, application and elution from the resin and concentration stages

The individual recoveries for each stages were negligible and the net recoveries from stage one through to stage four were within the acceptable range. The problem of the variable recoveries of basic amino acids especially lysine was (±11%) also as noted by Moore and Stein (1951) was also found in the present study.

As a formal statement of the individual recoveries found for solutions A2, B and B1 were 98.4%, 98.9% and 98.7%, respectively, relevant data for quintiplate analysis are given in Table 97, and these contrasted with the figure of 94.6% found by Siddiqi (1981).

For reasons discussed elsewhere the amino acids arginine, cysteine, histidine and methionine were not included in the present study.

Choice of Desiccants (Stage 5)

Normally little thought is given to the choice of dehydrating agents used to remove the final traces of moisture. However, certain adverse experiences in the present study have indicated that in this context the choice of desiccant is of more than normal importance.

Although Siddiqi (1981) reported that the use of concentrated sulphuric acid as a desiccant was suitable for honey amino acid concentrate, several other compounds such as phosphorous pentoxide, sodium hydroxide and potassium hydroxide were examined for suitability. This evaluation was undertaken because preliminary experiments had shown that the honey amino acid concentrate became discoloured into a blackmass in the presence of concentrated sulphuric acid and under conditions which precluded accidental spillage. In a few samples of foreign origin predominantly Mexican it was noticed that these were

TABLE 97

Average recovery of thirteen synthetic amino acids after quintiplate analysis of amino acid solution

No.	Amino acid solutions (10 mls) or honey sample (10g)	Addition of S1* (mls)	IXC[1]	AAAA[2]	Percentage recovery# (%)
1	Solution A	-	-	+	101.3
2	Solution A1	-	-**	+	98.8
3	Solution A2	-	+	+	98.4
4	Solution B	-	+	+	98.9
5	Solution B1	0.6	+	+	98.7
6	U.K. survey sample Code No. 174	0.6	+	+	99.0
7	China Light Amber Code No. 211	0.6	+	+	99.5
8	Mexico Yucatan Code No. 227	0.6	+	+	98.4

For notes to key *, **, 1 and 2 refer to Table 6.

- The average percentage values quoted are those of the average recovery percentage values obtained after quintiplate analysis of the 13 amino acids of amino acid solutions given in Table 6. The concentration measurements and percentage recovery for these amino acid solutions are given in Tables 13 to 29.

stored *in vacuo* in the presence of phosphorous pentoxide the whole concentrate became purplish-red far removed from their original colour. This may be due to volatile matter being present interacting with and being dehydrated by phosphorous pentoxide. For these reasons neither concentrated sulphuric acid nor phosphorous pentoxide should be used as desiccant under vacuum condition for honey amino acid concentrates.

This unexpected dehydration and other complex series of reactions could involve the sugars of honey or the catalytic action of polyphosphoric acids produced on the surface of the excess phosphorous pentoxide (Partington, 1961). Due to the risk of pollution by dust and other adverse environmental factors present in the laboratory atmosphere many precautions were taken to prevent such contamination from taking place; for example, no honey sample was left uncovered and no vessels were left unstoppered when not in use. Additional precautions were: covering the reaction vessels with parafilm, evaporation under reduced pressure and the use of a thoroughly cleaned desiccator with trap to prevent dust entering when re-pressuring. It was observed that when concentrated sulphuric acid was used for preventing access of ammonia to the closed vessels used for storing paper chromatograms, this dehydrating agent became discoloured after a period of about seven days. This could be one aspect of the phenomenon mentioned above. However, it was found that no such problems were encountered with either sodium hydroxide or potassium hydroxide used in the pellet form. As a matter of choice sodium hydroxide was used in the present study. At the end of the desiccation stage it was essential to have the dry residue concentrated in one area of the vessel and not spread over all available surfaces.

Re-constitution (Stage 6)

A bacterial resistant media - propan-2-ol/HCl mixture was used and steps were taken to ensure that the dry residue was uniformly wetted and transferred. Also, that there was no loss of small volumes used elsewhere on the glass surface. It was essential to ensure all glassware used in the extraction and transfer were completely grease free.

Storage (Stage 7)

The use of bacterio-stat media at temperature of 0^{o} to $4^{o}C$ was sufficient to prevent bacterial or mould growth. Frequent visual checks were made and no deterioration of the extract was noted.

Paper Electrophoresis and Chromatography (Stage 8)

The combination of paper electrophoresis and chromatography technique enabled greater sensitivity in the detection of low concentration ninhydrin positive substances and greater selectivity amongst the major amino acids. However, the effects of the residual levels of ammonia in the final extracts were evident in the slight discolouration of the background of the amino acid maps and the need to wait overnight for some of the low concentration spots to appear. Sometimes the effects of ammonia were compounded further by presence of ammonia generally found in analytical laboratories. Even with great care some amino acid maps were adversely affected and the relevant extracts were re-examined. Usually the mild effects often resulted in the backgrounds of the amino acid maps becoming pink after staining, especially when the laboratory temperature conditions were high, between $22^{o}C$ and $28^{o}C$. The faint spots which resulted due to low concentrations of ninhydrin complexes were sometimes difficult to distinguish from the usual pink background. The paper electrophoresis and chromatography

experiments were repeated at low laboratory temperature conditions between 15°C and 20°C, for comparison. Also, the recognition of closely occurring or overlapping ninhydrin complexes presented problems at first but were overcome with experience. This was the case for isoleucine-leucine, methionine-valine, threonine-glutamic acid and sometimes arginine-lysine. The cluster of ninhydrin complexes serine, aspartic acid and asparagine were not always separated but usually their boundaries could be outlined. If hydroxyproline was present, then if this ninhydrin complex was not recognised by its characteristic yellow coloured complex similar to that of proline in the first hour or so after staining, then its presence would usually go undetected. Therefore, the amino acid maps were checked at intervals of approximately fifteen minutes for the first three hours after staining with the ninhydrin reagent. Further, the R_F values for the ninhydrin complex spots were not calculated as there was variation in the movements of the spots relative to the solvent front. It was observed that in high laboratory temperature conditions, the solvent front had migrated very near to the top of the paper whilst under cold temperature conditions the solvent front travelled only two thirds of this distance. These conditions resulted in improved and impaired resolutions, respectively. Also cold temperatures impaired staining and it took up to two hours for some of the coloured complexes to appear. Some such as glucosamine, glutamine, β-alanine, α- and γ-aminobutyric acid and the two unidentified spots 'p' and 'x' usually took one to three days depending on their concentrations to become sufficiently visible to be outlined. Others such as pipecolic acid and hydroxypipecolic acid if present in low concentrations usually took a day before the spots became visible. Whereas in the case of Australia banksia sample No. 194, hydroxypipecolic acid was present in large amounts and became visible only two hours

after staining. This spot overlapped with the surrounding spots of proline, threonine and glutamic acid as shown in Figure 20. The entire technique of paper electrophoresis and chromatography, staining and the identification of ninhydrin spots required about one week per sample, although more than one sample per week could be examined.

It became evident from amino acid maps that asparagine, glutamine and tryptophan were also present in the final extract. It is likely that o-esters of serine and threonine, and other compounds such as amines, amino sugars and peptides may be present. Attempts were made to determine the identity of the unknown ninhydrin complex spots 'p', 'x', ξ, 'δ', 'ζ', 'ε', 'π', 'ω', 'λ', 'θ', 'σ' and 'Ω' detected on some of the amino acid maps. By comparing the relative positions of the ninhydrin positive substances on the amino acid maps obtained in this study and with those of Siddiqi (1981), it was thought that the identity of the unidentified ninhydrin positive substance labelled as 'ω' could be kynurine. When co-developed with standard kynurine the identity for the spot 'ω' and also for other unidentified spots could not be confirmed. Similarly, small amounts of other reference compounds were procured for similar studies and these compounds included kynurine, citrulline, ornithine and β-aminobutyric acid. The relative position of these reference compounds did not correlate to those of the unknown and their low levels precluded extraction from the paper chromatograms and subsequent total hydrolysis to confirm or otherwise their peptide nature. Moreover, the identification was further compounded by the fact that these unknown ninhydrin positive substances were always present in low concentrations estimated visually to be about 0.5 nMoles. Hence, the spot was observed to develop slowly after staining and the spots were very faint compared to the other identified compounds. At this low concentration level the trace curve peaks of those unidentified

ninhydrin substances would mearge into the baseline obtained on the autoanalyser chart recording and therefore none were detected.

Attempts to isolate some of these unidentified ninhydrin complexes from amino acid maps to which fifteen to twenty times the normal honey amino acid concentrates had been applied, were not successful. This was because the overloading not only resulted in overlapping of closely occurring spots but also streaking of all the spots due to overlapping and diffusion. Hence, the outlining of the spot in question was often difficult to judge. However, some of these ninhydrin complex spots were cut-out of the overloaded amino acid maps and eluted with acetone. The invisible residue obtained after evaporating off the acetone was subjected to the technique of mass spectroscopy as described by Siddiqi (1981). The result indicated that no compounds were present in these residues. The most likely explanation as to the identity of these compounds could be that they are peptides. Moreover, the presence of peptide like compounds were detected by performing the technique of paper electrophoresis and chromatography in the presence of pyridine. Those compounds that were separated on the paper chromatograms after complexing with ninhydrin reagent were yellow in colour and fluorescent when examined under ultraviolet light. The pattern of these separated spots could not be reproduced in a like manner to that of the amino acids.

Automatic Amino Acid Analyser (Stage 9)

From the present work and recent past reviews (Siddiqi, 1981 and Carter, 1983) on the world literature on the free amino acids present in honey, it has been reported that upto thirty two different ninhydrin positive substances have been identified to be present in honey.

However, some of these ninhydrin positive substances are very infrequent in occurrence perhaps in only one sample, for example, the unidentified ninhydrin positive substance labelled as 'Ω' found in Indian Gujarat 2 sample No. 225, and always in low concentration. Some of the remainder could only be detected semi-quantitatively by paper electrophoresis and chromatography technique and not all on a quantitative basis by the auto-analyser system employed. The remaining number of amino acids in honey approximated to the seventeen used routinely as standards to calibrate the technicon auto-analyser at the 25 nanomole level, corresponding to a chart peak height from 7 cm to 15 cm depending on the amino acid. When some of the honeys had been examined in a preliminary manner, it became obvious that four amino acids arginine, cysteine, histidine and methionine were not present in every sample and often if present gave tiny peaks just 0.5 cm above the baseline on the chart. At this level, about 2 nanomoles of amino acid, the measurements of the peak height and half peak width was grossly in error. This left some thirteen amino acids of reasonable concentration and chart peak size; although that for lysine was always small and not always present in every sample.

Due consideration was taken into account of the amount of final concentrate used in the auto-analyser for concentration measurements. Owing to the nature of the construction of the auto-analyser there are two columns and two resin filled cartridges for dispensing the amino acid solutions into the two columns. One cartridge is for the basic amino acids such as lysine, histidine and arginine and also a very large ammonia peak. The other cartridge is for the longer column on which the acid and neutral amino acids are separated and eluted in the order of elution. These amino acids are: aspartic acid, glutamic acid, serine, threonine, proline, glycine, alanine, cysteine, methionine,

leucine, isoleucine, tyrosine and phenylanine. The total amount of liquid added to each cartridge cannot exceed an upper limit of 60 µl, that is, a factor of three times that which was used for every sample loaded. Since sample loading of 40 µl gave decreased resolution and gross distortion due to high ammonia and major amino acids being off the chart scale. Hence, the particular upper limit with consideration for other amino acids was considered to be up to 30 µl per cartridge. Also, it was normal procedure to mix the amino acid solution with an internal standard norleucine of constant amount to compensate for variations due to instrumentation. Therefore, in the present study, it was found that by mixing 25 µl of the final concentrate with 25 µl of internal standard norleucine and then applying 20 µl of this mixture to each of the cartridges resulted in acceptable chart recordings.

In general the requirements of an internal standard are that it has a constant value and not normally present in the analysis. This is because it can be used to correct variations; for example, of pump diameter, of the extent of reaction of ninhydrin and other minor instrumental irregularities which could not be either predicted or closely controlled. In the above study norleucine is used and the response in terms of area under the eluted peak is calculated to the norleucine equivalent for all the chart sets. This has the advantage that it does appear at approximately the mid position and is therefore assumed to provide an average type of correction for the common amino acid pattern. However, in the case of honey where the amino acid pattern is not that of a typical protein hydrolysate the use of known artificial honeys of defined amino acid composition is essential. The possible use of other pairs of internal standards such as arginine for the short column and cysteic acid and tryptophan for the other column. These would appear at the extremes of the elution pattern which is

desirable but not possible. This additional control with paired internal standards was not possible in the present study.

Considerations were given to the use of electrical amplification to boost the height of small peaks on the baseline. This, however, resulted in the amplification of not only the peaks in question but also the baseline. This generalised baseline or noise could not be significantly reduced due to its operation in a general laboratory and also to instrument age.

The average percentage recovery value obtained for the thirteen amino acids of solution A applied to the auto-analyser before ion-exchange chromatography can be explained as due to two factors. One that the moisture content and the homogeneity of the amino acid solids used in the preparations of solutions A, B and S1 could not be ensured. This is in contrast to the specially purified dose samples supplied in sealed vials by the commercial firm. For reasons already explained, the standards containing 25 nanomoles of some seventeen amino acids would not be suitable to prepare artificial honey solutions of similar composition to the real sample. The other reason could be due to human factor in the normal operational procedure practised by the technician in the use of the auto-analyser which was not in continual use, especially as there were never sufficient departmental samples to ensure maximum efficiency and the down time was considerable.

The auto-analyser column on which the basic amino acids lysine, histidine and arginine are separated also eluted a very large peak for ammonia in spite of the precautions taken to eliminate its presence in the final extract. The conversion from a 7M ammonia solution to a residual of 10 μmoles in the final extract proved to be impossible to achieve and therefore the residual ammonia always present adversely

affected not only the accuracy of estimation of lysine but also the detection of histidine and arginine. Fortunately, however, the amino acids arginine and histidine were found to be present only in low concentrations and not in every honey sample by the technique of paper electrophoresis and chromatography. It was estimated that arginine was present at about a concentration of approximately 2 nanomoles or less. For this reason the peak for arginine was hardly ever seen on the chart recordings. Histidine and to some extent methyl-histidine were identified by Siddiqi (1981) and in the present work by the technique of paper electrophoresis and chromatography. This histidine level was always low and was detected by this technique in perhaps one quarter of the samples and methyl-histidine was comparable. Histidine rarely could be identified in the auto-analyser charts and the situation for arginine has already been mentioned. Furthermore the peak for lysine was far removed from the ammonia peak as compared against the histidine and arginine peaks occurring closely on either side of the ammonia peak. Lysine was found to be present in many of the honey samples and could also be measured quantitatively. The error of estimation for lysine was found to be ±11.0%.

Another reason for the relatively low concentration levels of these basic amino acids in the final extracts could be attributed to their R-groups. As it can be seen from the pI_R values given in Table 98 the degree and extent with which the R-group of each basic amino acid is likely to effects its binding and elution from the resin. Hence, arginine with a pI_R value of 10.77 will be followed by lysine of pI_R value 9.31 and finally histidine of pI_R value 7.59. These basic amino acids would tend to adhere to the ion-exchange resin and it was for this reason the ammonia solution concentration was raised over that used by Siddiqi (1981).

TABLE 98

The pI_I and pI_R values of the amino acids

Amino acids	pKa_1 ($\alpha COOH$)	pKa_2 (αNH_3^+)	pKa_3 (Rgroup)	pI_I^*	pI_R^{**}
α-Alanine	2.34	9.69	-	6.02	-
β-Alanine	3.60	10.19	-	6.90	-
α-Aminobutyric acid	2.55	9.60	-	6.08	-
γ-Aminobutyric acid	4.23	10.43	-	7.33	-
Arginine	2.17	9.06	12.48	5.62	10.77
Aspartic acid	2.09	9.82	3.86	5.96	6.84
Glutamic acid	2.19	9.67	4.25	5.93	6.96
Glutamine	2.17	9.13	-	5.65	-
Glycine	2.34	9.60	-	5.97	-
Histidine	1.82	9.17	6.00	5.50	7.59
Hydroxyproline	1.92	9.73	-	5.83	-
Isoleucine	2.36	9.68	-	6.02	-
Leucine	2.36	9.60	-	5.98	-
Lysine	2.18	8.95	9.67	5.67	9.31
Methionine	2.38	9.21	-	5.80	-
Phenylalanine	1.83	9.13	-	5.48	-
Proline	1.99	10.60	-	6.30	-
Serine	2.21	9.15	-	5.68	-
Threonine	2.63	10.43	-	6.53	-
Tyrosine	2.20	9.11	10.07	5.66	9.59
Valine	2.32	9.62	-	5.97	-

The pKa values were obtained from Dawes (1972) and Dawson *et al.*, (1969).

$$* = pI_I = \frac{pKa_1 + pKa_2}{2}$$

$$** = pI_R = \frac{pKa_2 + pKa_3}{2}$$

On the chart recordings of the auto-analyser it was generally observed that low concentration amino acids were indicative of small broad curve peaks, whereas the reverse was true for amino acids present in high concentrations. Some slight overlapping of elution peaks was observed in samples for closely eluting amino acids on the chart recordings. These were: serine and threonine, glycine and alanine, and isoleucine and leucine. The average total recovery of these amino acid pairs were found to be consistent. The imino acid proline was present in very high concentrations in nearly all the samples examined. The eluted chart peak for cysteine was observed only in China buckwheat sample No. 209 but its presence in low concentrations was evident in many samples by the technique of paper electrophoresis and chromatography. The quantitative determination of amino acid methionine could not be effected since the measured peak height was observed to be below 0.5 cm from the baseline in most of the samples.

Pollens

The microscopical examination of pollens present in honey termed 'mellissopalynology' by Louveaux, Maurizo and Vorwohl (1970) was evaluated using the technique described by Sawyer (1981) and Louveaux, Maurizo and Vorwohl (1970). For reasons quoted by these authors the majority of the isolated pollens were distorted due to the process of diffusion and/or osmosis. It was partly due to this reason that the classical pollen analysis could not be applied. Also, attempts at some of the more difficult pollen analyses were not carried out since these methods required prior knowledge of botany which was not within my expertise. Further, the application of specialised methods such as acetolysis for pollen in peat are designed to remove that type of

contamination from pollen samples so obtained. Furthermore, acetolysis procedure results in the destruction of algae cells which characterise honeydew honeys, and therefore this technique is limited in this respect (Moar, 1985). If these methods were applied to the already fragile pollen grains then these grains would sustain further damage thus reducing the possibility of identification. Furthermore, in view of the large number of samples that had to be examined to verify or otherwise the casual observations by beekeepers as to source. It was essential to use a simple reliable method such as that recommended by Louveaux, Maurizo and Vorwohl (1970) and hence this was done. A similar but simplified method was advertised and advocated at a summer school at Cardiff 1981 for the purpose of pollen identification.

The method outlined in the present work utilised basic fushin stain to enhance recognition and then eventual identification. The contents of isolated pollen ranged from that of almost no pollen to that which required two or three microscope slides to contain the pollen. The degree with which pollens from different genera were stained became apparent very early. For example, pollen as those of *Eucalyptus*, *Loranthus*, *Pinus* and generally of Myrtaceae family were difficult to stain. Pollen grains of *Trifolium*, *Vicia* and *Melilotus* stained lightly whereas those of *Brassica*, *Epilobium*, *Calluna*, *Castanea*, *Tilia*, *Prunus/pyrus* and some of the unidentified pollen of some foreign honeys stained with relative ease.

The identity of different species of pollen in a particular genus could not always be distinguished since perfect pollen from flowers concerned were unavailable. It was noticed that pollens of *Aesculus*, *Castanea* and *Lotus* were particularly troublesome as these have similar features. This was also evident for pollens of *Echium* genera in the

foreign samples. Therefore, in order to prevent confusion the identified pollens were quoted by their genus, as given in Table 7 and Appendix VIII.

The classification of identified pollen grains counted in each sample was according to that suggested by Louveaux, Maurizo and Vorwohl (1970). The counting of 1,200 grains per sample as recommended by U.K. and EEC regulations where triplicate counting is required was not carried out. This was because of the small amount of samples sent, there was sometimes only sufficient material for one amino acid extraction and one pollen estimation and also because of the time factor. Further, it was considered normal practice to re-evaluate pollen grains on a microscope slide if doubt regarding pollen content was experienced. Such doubts were either low pollen count for visually estimated high pollen content slide or an adverse computer report. However, since only limited counting was carried out the average percentage values obtained for the frequency classes were not adjusted for any digit after the decimal point. The accuracy of the expressed frequency class percent is likely to be in error with a range of not more than 2% to 4%.

This situation was the same but somewhat complicated for foreign honeys as they were sent and known only by their commercial description or port of departure. Most of the commercial samples had been collected from wild and/or semi-wild colonies by unskilled labourers so that the true knowledge of the major floral species could not be obtained. The only retrospective guideline was that given by Crane, Walker and Day (1984). The major species were indexed but this work did not appear till all the practical work and most of the thesis had been completed.

This comment could be remedied if every country had an efficient bibliography and documentation of floral souces. Although the International Bee Research Association is moving towards such an ideal this is a long way from realisation and it is another reason for having a data bank of floral sources and full composition available at sometime in the future.

Procurement of Experimental Hive on University Campus

At the onset of the present investiation period it was thought desirable for reasons listed below that there should be an acquisition of an experimental hive sited on the University campus. Such a hive would have enabled the study of:

1. The effects of environmental and seasonal changes on the composition and production of honey over a period starting from 1981 - 1984.
2. Whether the secretions of honeybee have a significant effect on the overall ninhydrin positive substance composition of honey.
3. Whether the ninhydrin positive substances of pollen injested contribute to the final ninhydrin positive substance composition of honey extracted.
4. To what degree and extent does the feeding of pollen supplement contribute to the final ninhydrin positive substance and also pollen composition of extracted honey.
5. Feeding a sugar product containing known amounts of amino acids and determining the ninhydrin positive substance composition of honey thus produced.

6. To establish the variation, if any, of the floral sources visited by honeybee from nectar deposited in cells and of combs during and at the end of the season, similar to that reported by Adams, Smith and Townsend (1979).

7. Whether the pollen obtained from pollen traps contribute amino acids to an amino acid free carbohydrate solution (33% glucose plus 33% fructose) and/or a honey of known amino acid composition over a period of one to two years. This latter item can be devisable because of the previous work of Carter (1983).

However, this was not done because suitable sites for a hive on campus were investigated over a period of years, but proved very difficult as the urbanised neighbourhood raised questions about liability if passersby or students were stung. The very few possible sites were also rendered impractical either by lack of bee access or forage or open to vandalism. The Head of Department, and the University could be separately and collectively liable in any legal action and the authority were naturally prudent against any future adverse circumstances.

Computation and Statistical Analysis

Compilation of all the data gathered for each honey sample in an orderly fashion into an SPSS data file required maticulous care and attention. Execution of the statistical analysis and interpretation of the results obtained demanded prior extensive specialised knowledge in statistical methods in relation to computer programming. This was overcome with the help of a statistician, Dr. Pemberton of the University Mathematics Department. It was upon his recommendations

that the following modifications and improvements were carried out and adhered to throughout the present analyses. These were:

1. the removal of groups containing less than ten sample numbers;
2. the transformation of the individual amino acid concentration measurements into their square roots and natural logrithms, and
3. the random selection of samples for estimation of misclassiciation rate.

The accuracy of the predicted classification results obtained from the SPSS increased as the number of samples per group increased, that is, from ten onwards. Modifcation of the amino acid data, that is, square root transformation of the concentration terms rather than natural logrithm transformation enabled the minimisation of the effects of sample variation and to stabilise variation in the amino acid data, itself.

From a biological view point there should be variation in the amino acid data due to the following factors even if in the unlikely event of a continuous supply of one floral source was available. These factors are: a single cell of a comb, all the cells of a comb, all the combs from a super, all the combs from a hive and the combination of several hives. The real life situation is more complicated due to the free ranging nature of the foraging bee and the instinctive use of floral source at her whim. The pattern of visitations to a particular floral source is maintained over a period of several hours. One should also consider the effects of manipulation of honey during extraction and processing followed by storage leading up to the time of analysis and the effects of the analysis on the amino acid composition. It was because of this reason that statistical calculations such as "student-t" or the "Hotterlings-T" were not performed.

Floral Source

Pollen

The initial attempt at discriminating between nine pollen groups using the SPSS indicated the need for amino acid data modification such as that given in Appendix IV(b). The outcome of such an analysis, refer to Table 63, suggested the need for statistical expertise and upon the suggestions of Dr. Pemberton the modifictions and improvements given above were utilized. The effects of such modifications and improvements resulted in the maximisation of the minimum Mahalanobis distance between groups, refer to Tables 64 and 65 and Figure 28.

However, due to the non-uniformity in the number of samples in the five pollen groups, refer to Table 64, to some extent influenced the success rate of the classiciation of the honeys. Most of the misclassified samples agreed as to the pollen content for which they were predicted to contain by the SPSS. This indicated that the SPSS predictions are accurate to some extent in evaluating the floral source of a honey sample. The accuracy with which these predictions can be relied upon is determined by two factors:

1. the re-definition of the unifloral pollen subclass group to contain pollen 90% and over, and
2. the inclusion of fifty or more unifloral samples per pollen group.

The reason for the remaining misclassified samples did not contain the pollen they were predicted to comprise of by the SPSS could be due to the similarity in the amino acid concentration measurements of the misclassified samples with those of the predicted pollen group. This similarity in the amino acid concentration measurements could be the product of:

1. honey gathered from several hives each of different unifloral and/or multifloral sources, and
2. honeybees from a single hive foraging pollen from one plant and nectar from another plant species.

It was noted in the present study that the replies received to questionnaire question 4 differed significantly in nearly all the samples from the microscopical observations by the technique of pollen analysis. This difference suggests that many of the honest observations of the beekeeper of an individual honeybee flights to specific flowers could be misleading as to source when whole frames or hives representing many thousands of flights are examined. The replies to questionnaire question 4 have been given in Tables 31, 34, 37, 40, 43, 46, 49 and 52 but the name and addresses of participant beekeepers have not been disclosed so as to retain confidentiality. In addition, two added complications should be considered:

1. that of merging of early spring and autumn harvests if only one collection per year is practised as was the case with some of the participant beekeepers, and
2. that of fascination of some crops such as *Brassica* or *Tilia* or *Calluna* for the honeybees could result in misleading observations even if a good crop of *Trifolium* may occur near the hive.

The use of discriminant analysis available on the SPSS package was successful to some extent in differentiating between the three pollens, that is *Brassica*, *Trifolium repens* and *Castanea* which were found to be common to following countries. These were: Canada, China, France and the United Kingdom. The significance of the results obtained in Table 96 can be given to question because there were only two samples

available for analysis in each of the first three countries. On the basis of this result, the findings do not agree with those of Kanematsu *et al.*, (1982) who used amino acid ratios to distinguish between honey samples from different countries but contained the same pollen species. Further, the amino acid ratios utilized by Siddiqi (1981) with some success in her analysis were also applied with other combination ratios in the interim report 1981-82 sent to the beekeepers during this study period. However, in view of the large number of honey samples and the instability of the variance-covariance matrix of the amino acid ratios during computation precluded their use for SPSS predictions.

Nectar or honeydew

A literature survey showed that the amino acid composition of honeydew as compared to that of nectar was generally reported to be higher both in terms of number and concentration of amino acids. Therefore, it is obvious from this that the honey amino acids could indicate its possible honeydew or nectar origins provided that a wide range of samples, seasonal and yearly variations of many specimens of honey provenance and furthermore, that the increase in honeydew honeys consistently exceed these other seasonal variations of the nectar honeys. Such an ideal state is not yet possible. However, statistical analysis of honey from the two limited groups resulted in the successful discrimination, refer to Table 89. However, due to the available samples in both these groups numbered ten the SPSS classification can be utilized as indicative of the usefulness of the chemical technique in distinguishing between honeydew and nectar honeys.

Geographical Source

When discriminating honeys from different countries it would have been ideal if authentic verified reference samples were first statistically analysed. This would then be followed by statistical verification of unknown or suspect samples with the reference honeys. However, due to the relatively limited number of foreign honey samples available for analysis of which reliable information as to source were available only for one or two samples. These were used for reference samples in default of verified official samples mentioned above which were not available. Moreover, the authenticity of these reference samples could not be verified with certainty as to the country they were alleged to have originated. Nonetheless, with the available samples the predictive classification results obtained by the SPSS as shown in Tables 83 to 88, 92, 95 and 96 indicate that the honeys were classified on the basis of the floral origin of the samples rather than geographical source.

The statistical results obtained from the SPSS programme in the present work suggests that there is a need for investigation of all the factors mentioned above and elsewhere in this thesis. Thereafter, the predicted classification from such an SPSS programme could be effectively used by a Public Analyst to determine the origins of a honey sample. Moreover, as suggested before, a data bank containing many unifloral honeys for the SPSS programme howsoever modified so as to provide a genuine useful searching and indexing system giving highly accurate predictions and fulfilling its undoubted potential.

The boundary between aspects of assessment and evaluation were somewhat arbitary. In general the self criticism and likely errors are dealt with in this assessment section. Whereas the evaluation of

the present results are reserved for the following section, 'Evaluation of Present Work'. Where necessary the relevant literature was quoted.

EVALUATION OF PRESENT WORK

Proteins

At the time of honey protein investigation, literature survey showed that the proteins had not been used for characterising honeys. However, the correlation of honey protein types with geographical source which is of course one of the present aims but was not possible because of the present conditions. The American authors Reeder, Richie and Guenther (1986) accomplished this objective only by using highly sophisticated and expensive technology which included techniques such as ultra-thin layer isoelectric focusing, laser scanner and chemometrics programme on mainframe computer. Also, this technology was applied to a geographically favoured and contained region. However, this form of technology was not possible at Salford and a less expensive but more restrictive survey was carried out on raw honey and protein concentrates prepared by ultrafiltration. This was followed by examination using SDS-PAGE on cylindrical gels.

The concentration of proteins in the honey concentrate obtained after ultrafiltration and then freeze-drying was determined by the Lowry *et al.*, (1951) method. The average value of 172 mg of protein per 100g of honey was obtained from nine samples of honey examined. This average value compares suitably with that of 169 mg per 100g of honey reported by White and Rudyj (1978). Dialysis and ultrafiltration being the two most common methods employed to date for honey protein concentration were compared by White (1978), to determine the loss of nitrogen components during the period of operation of these two

procedures. He reported that the ultrafiltration technique employed by Bergner and Diemar (1975a) using filter membranes to retain molecules of 10,000 daltons and above, encountered loss of nitrogen components of between 33% - 45%. White (1978) calculated that 55% of the soluble filterable nitrogen components were lost by the ultrafiltration procedure used by Paine *et al.*, (1934). However, the dialysis technique used by White and Kushnir (1967b) was estimated to have losses between 40% - 60% as a percentage of total soluble nitrogen components. In the present study the loss of filterable nitrogen components during ultrafiltration were indicated to be comparable with those encountered by Bergner and Diemar (1975a). Therefore, the use of a filter membrane with calibrated pore size can be employed to determine the molecular size of proteins present in honey.

It was not known at the time of the present honey protein analysis that Bagdanov (1981) had used the Bradford/Bio-rad protein-dye binding technique to determine protein concentration in honey. It would have been of value to have assessed the suitability of two such diverse methods of protein determination using the nine samples available at the time of the investigation. This comparison was not evaluated later on during the research period because of the aim to utilise chemical analysis to determine source and it was found that after three months work that the proteins were not uniquely defined. This has been discussed in the previous section. Therefore, the emphasis changed to an examination of the free amino acids. However, the average value of 129 mg of proteins per 100g of honey reported by Bagdanov (1981) is rather low compared to that reported by White and Rudyi (1978) and to that found in the present study.

Protein Band Patterns

The protein band pattern obtained on the SDS-PAGE gels to which raw honey had been applied was consistent in the fifteen commercial samples examined. The band pattern consistency observed on the heat treated sample No. 261 (English Chorley honey) and also for the remaining fourteen samples indicated the likelihood of denaturation and/or coagulation of the proteins present in these samples. The lack of providence on the prior treatment of these fourteen samples before receiving them indicated that heating or pasteurisation at an industrial level presents a barrier not only to source of geographical identification but also to the determination of enzymes present in honey. Moreover, the unusual protein band pattern observed on the 10% SDS-PAGE gels of the national honey show sample No. 252, Figure 4, as compared to those observed in Figure 3 could be due to two reasons:

1. that this sample No. 252 had been subjected to uncharacteristic heating for the purpose of clarification with probable addition of flavours so that such honey, of transleucent quality and delicate flavour, can be presented for competition at events such as National honey shows. This possibility could/could not be verified because if the outcome was true then this would implicate the owner in malpractice for financial and reputable advantages, or
2. more likely that honeybees were fed on a pollen substitute such as soya bean protein. This possibility was sought, in this case the unusual bands were not due to soya protein.

These results of honey proteins on PAGE gels were presented as a poster session at the 'Fifth Meeting of the International Electrophoretic Society' in London (1986).

Other unusual proteins that have been noticed are the proteins associated with thixotropy in heather honey and these have been evaluated briefly by Pryce-Jones (1944) and more recently at Salford. Current work (Hamilton, 1987) shows that proteins which migrate as one peak on sephadex with a molecular weight of about 50,000 was infact resolved by gradient pore electrophoresis into two closely associated proteins of similar weight. Also other unusual proteins can occur being dependent on the exposure of the bee or honey to unusual conditions.

Enzymes

Generally, the proteins of normal honey constitute less than 1% of the total composition of honey (White *et al.*, 1962). Thixotropic honeys predominantly heather and manuka have additional protein (1.5%) (Pryce-Jones, 1944) and these specific proteins have infact been characterised as mentioned above. It is because of this low level of proteins and consequently even lower levels of active proteins namely enzymes that their detection in honey demand an elaborate, maticulous and lengthy procedures. These procedures are necessary to ensure adequate quantities of active enzymes since honey enzymes have been shown to display unique and unusual characteristic properties compared to similar enzymes from other sources catalysing the same substrate. The honey enzymes under investigation, described in general in Table 4 were: diastase, glucose oxidase and invertase. (White, 1952; White and Maher, 1953; Schepartz and Suber, 1964 and 1966; White, 1966; Rinaudo *et al.*, 1973(a) and (b) and Takenaka and Echigo, 1975). Further, the presence of these active enzymes in honey would be expected to be minimal due to the commercial processing of honey. Hence, in that connection the presence of hydroxymethylfurfural would be a crude

indicator of heating. Therefore, a special honey was obtained by the courtsey of Dr. Croft whose sample - English Chorley honey Code No. 261 had not been heated so as to minimise loss of enzyme activity. Suitable comparisons could not be made with commercial honey enzymes because these were not available. Hence, comparison had to be made using standard laboratory enzymes of different characteristic properties. As expected enzyme activity was observed, except for glucose oxidase, on PAGE gels to which standard laboratory enzymes were applied. Such activity was not observed on gels to which only honey was applied. The reasons for this have been discussed in the previous section.

The main aim of the present work was to develop a chemical method which would adequately characterise even a pollen-free honey as to source. For this reason, the major components of honey such as carbohydrates were not investigated. However, constituents of honey present in small amounts or trace amounts were considered worthy of investigation. Proteins were initially selected for this purpose and the results indicated that the native proteins were too susceptible to heating and other pre-treatments to be of value in the present context. Therefore, interest centred on amino acids for both floral and geographical determinations.

Ninhydrin Positive Substances

The range of compounds within this overall title with the exception of amino acids could not be examined quantitatively and were present in very low and erratic amounts.

Ion-exchange Chromatography

The viscous nature of honey demanded the need for a large mesh size resin which after removal of fine resin particles followed by regeneration from its Na^+ form to the H^+ form was found to be suitable for use on honey.

The lower but respectable recovery values (94.6%) reported by Siddiqi (1981) for the thirteen synthetic amino acid mixture as compared for the same thirteen amino acid mixture (98.6%) obtained in the present study could be due to:

1. Her not using 7M ammonium hydroxide solution for elution of the ninhydrin positive substances after binding to the Dowex resin as described by Bosi and Battaglini (1978) and in the present investigation.

2. The technique employed for the removal of excess ammonia. Siddiqi used a steam bath and pressurized air to facilitate rapid evaporation. However, this procedure was found to be liable to contamination in spite of precautions and was also prone to sample loss when regulating air flow. Moreover, this procedure was found to be slow compared to the enclosed low pressure evaporation method employed in the present analysis and which was not subject to sample loss or contaimination.

Further, Siddiqi (1981) did not assess the effects of the predominant sugars of honey namely glucose and fructose, and also of the effects of honey constituents on addition of amino acids to honey. However, she assessed the effects of glucose (20% w/v) as 'having no adverse effects on the amino acid analyser results'. Moreover, in assessing the effects of the predominant sugars of honey and honey

constituents on amino acids it was observed, Table 97, that these components of honey slightly enhanced the recovery of the amino acids from the Dowex resin.

Paper Electrophoresis and Chromatography

The method of paper electrophoresis and chromatography followed by staining was used for the qualitative determination of a wide range of ninhydrin positive substances not only those quantitatively determined by the auto-analyser but others not detected by the automatic instrument. However, the paper electrophoresis and chromatography technique was not utilised for semi-quantitative estimation of the detected ninhydrin positive substances as evaluated by Siddiqi (1981). For reasons already given in the previous section it was considered that visual determination of the amount of the ninhydrin positive substances could lead to both over- and/or under-estimation. Therefore, the method of paper electrophoresis and chromatography was used as an alternative to and/or in conjunction with pollen analysis to assess the origins of both pollen-free and pollen containing honey samples. Using the paper electrophoresis and chromatography technique Siddiqi (1981) was able to detect the presence of thirty three ninhydrin positive substances as compared against the forty separated from the honeys examined. Moreover, she identified the presence of β-aminobutyric acid, citrulline, kynurine and ornithine. The presence of these four ninhydrin positive substances could not be confirmed by co-developing with procured reference standards for reasons already given in the previous section. However, the reasons for this was due to biological variation and that the samples were not identical as discussed in the previous section. Hence, there can be no guarantee that these identified and/or unidentified ninhydrin positive substances would be

present in every sample and was in fact occurring very rarely, particularly in individual samples.

Automatic Amino Acid Analyser

With biological fluids such as honey containing different types and amounts of ninhydrin positive substances it is possible that the presence of amides, amines, amino sugars and small peptides, could co-elute with any of the other thirteen amino acids. This was particularly noted by Davies (1975), in that, the asparagine, glutamine, threonine and serine eluted into one peak. This was not observed with the auto-analyser system employed in this work, although the threonine and serine elutions often overlapped to some extent but these peaks could be clearly distinguished. Davies (1975) carried out direct application of the diluted honey solutions to the columns of the amino acid analyser. After calibration, Davies was able to detect the presence of twenty two amino acids and seven unknowns. This direct application of diluted honey was not carried out because it was feared by the technician operating the auto-analyser system that honey constituents such as spores, yeast, pollen, dust and similar large inclusions present even after clarification could block the columns and tubings of the analyser. The amino acid analyser used in the present study was calibrated to detect the following seventeen amino acids on a two hour programmed cycle. These amino acids were: lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine and phenylanine, in the order given. For this reason and those quoted in the previous section quantitative measurements were determined for only thirteen amino acids with the exclusion of

histidine, arginine, cysteine and methionine.

Pollens

The diversity of the floral sources utilized by the honeybees in the British Isle for the production of honey can be seen from the variety of different pollen genera identified in the U.K. survey samples. The major and rare pollen types identified in this study are in good agreement with the major floral sources of honey as listed by Crane, Walker and Day (1984), and those given by Sawyer (1981), refer to Appendix VIII. The occurrence of pollen such as *Brassica*, *Trifolium*, *Eucalyptus*, *Cirsium* and *Melilotus* was evident in most of the samples examined. The presence of pollen which are not common to the country from which the honeys were stated to have originated was evident in three commercial samples namely MR68 (Code No. 243), English pure (248) and English clover (254). These three samples were described as of English origin and their pollen content agree with those identified by Sawyer (1975) in honeys that had been imported from other countries of the world.

Statistical Analysis

The transformation of the amino acid concentration measurements utilised by Gilbert *et al.*, (1981) and Davies and Harris (1982) was not carried out in this study because it did not result in the successful stabilisation of the variance-covariance matrix. This transformation was the dividing of the individual amino acid measurement by the total content and then multiplying by the number one thousand. However, the square root transformation adopted in the present analyses stabalised the variance-covariance matrix and coupled with pollen analysis enabled

the correct classification of honey samples without the ommission of outliners as reported by Gilbert *et al.*, (1981). Other overall modifications such as:

1. the redution in the number of sample groups,
2. the defining of unifloral pollen subclass, and
3. the random selection of samples per group for predictive classification and estimation of misclassification rate.

These modifications vastly improved the maximisation of the minimum Mahalanobis distance, that is, discrimination between sample groups. However, the limited and inconsistent number of samples per group to some extent limited the predictive capability of the SPSS. The use of amino acid ratios was attempted as described in the British Beekeepers Association interim report 1981 - 1982 and also those used by the Japanese authors Kanematsu *et al.*, (1982) were not found to have any statistical advantage over the square root transformation. Therefore, the amino acid ratios were not adopted for statistical analysis in this study. Furthermore, Gilbert and co-workers used data in such a way that on a substantially standardised SPSS package, so as to produce defined boundaries round sample groupings in two dimensions. It should be pointed out that they used large groupings of unconfirmed floral sources and few experimental variables were considered. In the present study since all factors were considered and each sub-group was independently verified by pollen analysis. These factors lead to a multiplicity of small sub-groups and a consequent randomness of data, so that boundaries could not be drawn in the present work. To overcome the lack of boundaries the SPSS was heavily modified with consequential improvements in

discrimination but still not significantly to draw the 95% confidence boundary.

Nonetheless, the predictive classification and misclassification computed by the SPSS programme suggests that the honeys in this investigation were classified on the basis of floral origin of sample rather than geographical sources. These findings do not confirm with those reported by Gilbert *et al.*, (1981). The reason for this could be that Gilbert *et al.*, did not verify the floral origins of their samples by pollen analysis, but relied upon the description given to them by their donors. It is a well known fact that commercial labelling of honey containers has an eye for sales appeal and therefore, the labels can often deliberately mislead as to the true source of the honey in the containers; for example, English Penine Heather honey sample No. 251 was found to contain 75% *Brassica* pollen. This in turn could explain why some data of Gilbert *et al.*, (1981) were omitted as outliners. Also coupled with the instability of variance-covariance matrix that the U.K. samples were incorrectly classified by these authors for samples of Argentinian and Canadian origins, by their SPSS programme.

In the present study, the modified SPSS programme was also able to distinguish between the U.K. survey and foreign, refer to Table 94, and also between English and European honey, refer to Table 88. These findings were in conformity with those reported by Davies and Harris (1982). Further, the similarity of pollen composition especially between the English and European honeys seems to indicate that the composition of honey amino acids is determined to a large extent by its floral source. However, the lack of background knowledge related to each foreign honey would seem to disprove these findings. It was not possible to obtain official verified samples from research stations of each

country nor was sufficient time or resources available for such a programme to be attempted no matter how desirable.

Nevertheless, the present work embodied in this thesis indicates that the credibility of the SPSS predictions were governed by the number of samples available for a particular analysis, for example, the success of *Brassica* honey classification was due to the sample size. Further, in spite of the lack of adequate sample numbers per group the SPSS classifications gave every indication of the potential use and application of this chemical technique to various aspects of honey production.

It is suggested that with the increased use of computers there is a need to validate the application of this chemical technique as an official method. Before this can be done it is however, necessary to establish a reciprical relationship with two or more independent methods with those utilised in this investigation. A great body of evidence can then be compiled by various research workers in other laboratories collaborating with Public Analyst's and this would then justify the use of such a chemical technique.

ENVIRONMENTAL AND PROCESSING FACTORS AFFECTING HONEY

The following topics will now be considered individually and these are: Adulteration, Sugar-feeding of honeybees, Heating of honey, Seasonal variations, Major honey sources and agricultural practices in British Isles, Toxic honeys and Honeybee species.

Adulteration

It is known that English honeys command a high price in the honey market because it is considered as being unique. This has lead to the temptation of adulteration via adulterants such as commercial invert

and glucose syrups, high fructose corn syrup and cheap low grade foreign honeys. Various techniques have been developed by interested analysts to detect the presence of these adulterants in honey. The most commonly used techniques are:

1. Monitoring increased levels of hydroxymethylfurfural (HMF) and maltodextrin due to the addition of commercial invert syrup and glucose syrups, respectively (Serra and Gomez, 1986). It is known that adverse heating and storage conditions also gives rise to HMF levels in honey (White, 1980a).
2. The detection of blending honeys with high fructose corn syrup (HFCS) requires the use of an expensive technique namely Stable Isotope Ratio Mass Spectrometry (Doner and White 1977; White and Doner 1978). This technique can differentiate between C_3 and C_4 plants which employ different photosynthetic pathways. Honey is the product from C_3 plant secretions, whereas HFCS is obtained from C_4 plants (Edwards and Walker, 1983 and Prince, 1983).
3. The use of pollen analysis can reveal the presence of pollen not found in the British Isles has been reported by Sawyer (1975). However, the use of high pressure filtration by commercial producers enables the total removal of pollen. Thus, invalidating the use of pollen analysis on such honeys (James, 1969).

In the present study, pollen analysis of honey sample No. 204 (Canada Samantha Clover) revealed that no pollen was present. This sample was suspected to have been subjected to high pressure filtration. Further, the absence of pollen in sample Nos. 234, 235 and 236 were suspected to have been similarly processed. Both the quantitative and qualitative amino acid analysis of sample No. 204 revealed low levels

of amino acids as compared against other honeys of Canadian origin. Inquiries by Drs. Croft and Washington revealed that this particular honey was rejected by a supermarket retailer for its low grade quality and was purchased by another. It was suspected that sample 204 was then blended with a sugar based syrup whose identity could not be established with the available resouces and sold relatively cheaply compared to other foreign honeys. It is known from the present investigations and that of Davies (1975) that amino acid constituents of sugar syrups do not significantly affect the overall amino acid composition of adulterated honey. However, the amino acids concentrations levels are known to be reduced depending upon the amount of adulterant added.

Furthermore, the true origins of four English samples suspected of being adulterated with foreign honey was established with the aid of pollen analysis, amino acid maps and predictive classification by the SPSS. It was found that sample No. 243 was estimated to be one-half English and one-half Mexican, and sample No. 244 was also estimated to be one-quarter English and three-quarters Mexican. Whereas, sample Nos. 253 and 254 were found to contain pollen normally found in Australian honey but the amount of Australian honey added could not be established. All these four honeys were quoted as being English and were sold as such. These findings were later confirmed by the sample donors and court action by Public Analyst resulted in the successful prosecution of the commercial producers involved. This form of fraudulence has been termed as misrepresentation by Sawyer (1975). The possible presence of these foreign pollens in English honeys could be due to:

1. The result of unfortunate mixing with leftover honey in the machinery after a batch of honey from a particular country has been processed.

2. The fact that not all the pollens filtered out can be successfully removed from the filters.
3. The delibrate mislabelling for financial advantage.

In a sample of honey containing cut comb it was noted that honey from the comb and that in the jar were different. This was confirmed not only by pollen analysis and amino acid maps but also by the predictive classification by the SPSS, refer to Table 92. The honey in the comb was thought to have originated from Mexico and that in the jar from Australia. The identities of these sources could not be confirmed from the commercial producer.

These findings agree with that suggested by Siddiqi (1981) that honey amino acids can be used for determining the authenticity of suspected samples. Also, indications were noted that this chemical technique could be used to determine the degree of blending.

Sugar Feeding of Honeybees

The artificial feeding of honeybees with sugar syrup is normally carried out to compensate for deficiencies in available nectar source and for prolonged periods of bad weather (Croft, 1986). Although this is a recommended practice for beekeepers however, this can lead to temptations of overfeeding and the resultant honey can be classed as adulterated. Some of the U.K. survey honeys received were observed to be unusually 'thin and runny' in nature compared to other honeys. From the replies received to questionnaire question number 6 it was noted that most of this 'thin runny' honey had been the product of feeding sugar to honeybees. Further, it was considered that some of these 'thin runny' honeys could be the result of collecting unripened honey but this could not be verified.

The reason for the limited success in discriminating honey samples which were products of feeding sugar to honeybees and those that were not, was mainly due to the variation in amount and different types of sugar products utilized by the participating beekeepers, refer to Tables 70, 71, 72 and 73. This hindered the purpose of establishing the effects of feeding sugar syrup to honeybees on the amino acid composition of honey obtained from such colonies, refer to Table 74. It was known from the quantitative determination of amino acids of commercial sugar syrups in this study, Table 61, and from that of Davies (1975) that the feeding of sugar should not have a significant effect on the overall proportion of amino acids. Further, it was expected that the content of imino acid proline relative to the other amino acids should be higher in terms of concentration. This was evident in most of the sugar fed samples examined in this study but was noted to be dependent upon the degree, extent and type of sugar fed. This was reflected on the results obtained in Table 74, which was not as entirely expected. This evaluation was based on the findings of Bergner and Hahn (1972) that proline is added to honey in an amount by far the largest compared to the other amino acids by the *Apis mellifera* honeybee and later reported by Siddiqi (1981).

The general guidelines suggested by Croft (1986) on feeding sugar to honeybees is both to be encouraged and recommended. However, it is not considered worthy to recommend a product obtained from a colony to which 10 kg of sugar per hive is fed, although this is a recommended practice for autumn feeding, but to quote 'honey yield' of 20 kg of honey per hive. This would lead one to believe that such honeys are practically sugar with vastly reduced or no intrinsic value at all and probably closely akin to sugar syrup in composition. In this connection

the ready availability of high fructose syrup can offer temptations of illegal blending or addition of pollens to confuse the Public Analyst. Such practices if they become prevelant in the U.K. could be detected by amino acid composition or by very expensive mass spectral measurements.

Heating of Honey

The extent of heating honey is usually determined by following the rise in 5-hydroxymethylfurfural (HMF) content. However, discriminant analysis indicated that heating honey has some effect on its amino acid composition. The degree and extent of this effect could not be evaluated as it was noted from replies received to questionnaire question 5, the non-uniformity with which the honeys examined were heated. Further, in selective qualitative screening of random samples it was apparent that the U.K. survey honeys were subject to minimal heating. This was evident by the amount of colour formation with aniline as compared to that obtained with commercial samples. This was due to the nature of processing techniques to which honeys are usually subjected to at industrial level. The responses to questionnaire question number 5 suggest that most of the beekeepers adhere to the general recommendations of heating honey. However, it was noted that heat treatment at a temperature of 48.9C (120^{o}F) for some fifty hours X2 would not only result in a flavourless poor quality honey, but also reflect on the reputation of beekeepers in this country.

Statistical analysis of samples subjected to both heating and were a product of sugar feeding could not be evaluated with any certainty by predictive classification aspects of the SPSS. This was due to the inconsistency of heating and sugar-feeding and also of sample numbers per group, refer to Tables 75 to 82.

Seasonal Variations

Changes in seasonal climatic conditions and for that matter year to year, can influence the availability of nectar and to some extent pollen and their constituents. This in turn reflects on the production of honey and its quality (White, 1980c and Maurizo, 1980). It has been reported by Carter (1983) that the amino acid composition of honey could be a function of the amino acid composition of pollen ingested by the honeybee. He also pointed out that the contribution of the amino acids from pollen present in a jar of honey is negligible but significant when considering the amount of pollen that may be present in a hive. It was observed by Adams, Smith and Townsend (1979) that honey from different floral sources during seasonal changes may be present on a comb and that for the comb to become nearly unifloral an extended flowering period is required before the deposition of unifloral honey can be acheived on a comb. Similarly, seasonal changes can be prevalent on the production of honeydew and its constituents as reported by Mittler (1958), Noda, Sogawa and Saito (1973) and Hertel and Kunkel (1977).

Discriminant analysis of honey harvested in the calender years 1981 and 1982, did to some extent confirm the possibility of seasonal changes influencing the honey amino acid composition, refer to Tables 66 and 67. Also, it could not be established whether seasonal changes influenced the total amino acid content or proportion of the different amino acids present in honey. It was also noted from the available information regarding each honey sample of the U.K. survey that the conditions to which some of the honeys were subjected played an important part in the outcome of the statistical analysis. This can be overcome by obtaining honey from newly established control hives that have had no honey or

pollen stores and has a fresh brood chamber. Such control hives should be placed alongside existing colonies and both these should be carefully monitored with annual changes of the control hives over a period of perhaps eleven years attributed to the solar activity cycle. This would establish the pattern of seasonal changes influencing not only the production of honey but also its constituents for the major honey sources worldwide.

Major Honey Sources and Agricultural Practice in the British Isles

Referring to the major survey of honey sources in the U.K. carried out by Deans (1957) it can be seen from the predominant pollen types identified in the U.K. survey honeys that there have been a significant changes in the agricultural practices in the British Isles. These changes have been shown in Table 99. Comparing the list of pollens identified both in the U.K. survey and the foreign honeys as given in Appendix VIII it can be seen that there is good agreement between the pollens identified and the major honey sources catalogued by Crane, Walker and Day (1984).

Toxic Honeys

The most likely source of toxic compounds in honey could originate from the plant - nectar and honeydew or during extraction, processing and storage and also from the environment.

In the United Kingdom honey from nectars of *Rhododendron* plant or *Aesculus* especially *Aesculus hippocastanum* and *Tilia* such as *Tilia petiolasis*, *Tilia orbicular* and *Tilia tomentosa* are known to contain toxic compounds which have been reported to be injurious to both consumers - humans and honeybees. Nectar from *Tilia* and also the honey from these species are reported to be harmful to bees. Palmer-Jones

TABLE 99

Major honey sources in the United Kingdom.

Plant Species	Deans		Howells	Mistry
	England 1952[1]	Scotland 1952[1]	Wales 1969[2]	U.K. 1987[2]
Brassica	4	5	2	1
Calluna	-	4	-	4
Castanea	7	-	-	3
Myosotis	-	-	-	5
Prunus/pyrus	2	2	3	-
Rubus	3	3	4	6*
Trifolium repens	1	1	1	2
Vicia	8	10	-	7**
Aesculus	-	-	-	6

1 = Ranked as plant importance in survey

2 = Ranked as percentage of honey samples recieved

* = Ranked in position with *Aesculus* plant

** = Ranked in position with *Impatiens* and *Clematis* plants.

(1947), Howes (1979), Kerkvleit (1981) and Atkins (1982). Honeydew from limes has been reported to be harmful to bees. This is mainly due to the presence of physiologically harmful sugars (Crane, 1980).

Also honey from *Ligustrum* (privit) and *Senecio* (ragwort) are known to give rise to distinctly bitter tasting and strong aroma and flavoured honeys (Howes, 1979).

The presence of compounds introduced into honey during extraction, processing and storage can also contribute to the bitter taste or strong flavour of honeys. These compounds could be benzaldehyde and smoke used for repelling honeybees from comb or metallic residues resultant of storage in metal containers, (Hooper, 1976 and Morse, 1974). Honey with high phenol content has been reported to be bitter tasting (Campus, Madau and Solinas, 1983).

The use of chemicals to control pests and insects in the environment have also contributed to the increase in the mortality rate of honeybees. This is especially evident during and after spraying crops with such compounds contrary to the advice of Ministry of Agriculture and Food directives.

In the present investigation, there were four honeys - one U.K. survey sample No. 38 was described by its donor to have an obnoxious flavour and was suspected to contain compounds which could cause symptoms of poisoning or that of nausea. The other three honeys were U.K. survey sample Nos. 89, 90 and 91 and were described by their donor to have a bitter taste. The distribution of pollen in each of these four samples showed the presence of *Castanea* (Sweetchestnut) pollen. Further, the presence of other pollen such as *Ligustrum* (privit), *Heracleum* (Hogweed) and *Tilia* (lime) were also noted. Honeys obtained from such plants have been reported to give rise to bitter flavoured

honeys (Crane, Walker and Day, 1984).

The amino acid maps, pollen content and predictive classification by the SPSS indicated that U.K. survey sample No. 38 was similar in composition to that of U.K. survey sample No. 36 (same donor). Also, the U.K. survey samples No. 89, 90 and 91 displayed similarity in composition. However, the bitter tasting nature of these four honeys could not be confirmed because the present study was conducted in a building designed for radioactive research where such sensory examinations are forbidden. Further, such subjective evaluation is only reliable with considerable experience, a non-fatigued pallate and many similar samples collected at one time. Furthermore, such observations conducted by a tasting panel are not based on one individuals assessment. The detection and identification of the presence of compounds reported to occur in bitter tasting or strong flavour honey was not carried out. This was because such compounds would only be detected in very fresh honeys obtained within days of occurrence and examined for the presence of these compounds with the sensitive gas-liquid chromatography technique. This was not possible in the present investigation.

Honeybee Species

The low proline content of honey produced by *Apis cerena indica* compared to that by *Apis mellifera* was used by Davies (1975) as an indication of honeybee species. Amino acid analysis of four honeys received from the Indian sub-continent revealed that two samples Nos. 224 and 225 were of very low proline content and the other two samples Nos. 169 and 233 had proline content similar to that of honey produced by *Apis mellifera*. It is known that both samples, Nos. 224 and 225, were the product of nectar gathered by either *Apis cerena* or *Apis florea*.

On the other hand sample No. 233 was known to be an admixture with other products whose identities were not known. It was not known whether sample No. 169 was either an admixture or the result of nectar gathered by *Apis mellifera* or similar product to that sample No. 233.

SIGNIFICANCE OF PRESENT WORK

In view of the relative ease with which pollen can be removed from honey to comply with commercial interests. Therefore, as stated in the aims that there was a need to establish a chemical technique to identify the floral and geographical source of honey. In the absence of pollen a chemical technique such as that described here would become of valuable analytical importance.

Although the separation and patterns of proteins are a possibility for geographical and floral source, the resolution with present equipment precluded the use of proteins for this purpose. The present study is therefore concerned with predominant use of amino acids and their changing pattern with source.

Even though this is the first national survey which attempted to compare conventional microscopical evaluation - pollen analysis with the corresponding amino acid pattern. This correlation was not as close as desired due to the lack of unifloral source as this aspect is discussed elsewhere.

The feasibility of catogarising honeys using pollen analysis and then correlating the quantitative amino acid measurements by the application of statistical analysis using the SPSS proved successful in the identification as to source. It was established that the predictive classification aspect of the discriminant analysis was based on floral source rather than on geo-political boundaries. From the evaluation of the present

work it was concluded that the potential of such a chemical technique is still in the embryonic stage but it also has the capacity to be able to differentiate the various aspects of honey production. These aspects are: honeydew and nectar honeys, feeding sugar to honeybees, heating, commercial processing, adulteration especially with foreign honeys and floral sources of different pollen species belonging to the same plant family.

Further, the application of paper electrophoresis and chromatography technique in association with that of pollen analysis for the purpose of verifying the geographical source of a sample proved useful, especially for pollen-free honeys. Also, the application of this technique proved useful in distinguishing the following: between honeybee species - the Eastern honeybee *Apis cerena* and Western honeybee *Apis mellifera*; adulteration with foreign honey and to some extent sugar products, and honeys the result of moderate to excessive sugar feeding of honeybees.

A brief but concise account of the application of this chemical technique for use on honey was presented at the '1st Bee Research Workers Colloquia' in London, 1986. A report on this work was presented to the British Beekeepers Association and also it has been acknowledged by the International Bee Research Association, 1986.

FUTURE WORK

The following needs to be assessed and evaluated:

1. On the predictive classification and data handling aspect of the suitably modified SPSS programme, this can be used to full advantage if the following conditions can be achieved such as:
   (a) Obtaining honey samples of known provenance.
   (b) Obtaining large sample numbers per group, at least fifty.

(c) Using unifloral honeys containing over 90% pollen from one floral source.

(d) Performing quantitative determination of all the detectable and identifiable ninhydrin positive substances.

The advantages of ensuring these conditions, as deduced from the present work, would be to:

(i) Improve the potential of sub-group allocation followed by identification and correct classification of unifloral sources.

(ii) Ease the recognition of causes of misclassification.

(iii) Enable the characterisation of unusual ninhydrin positive substances with source both floral and geographical.

(iv) Produce statistically significant information.

(v) Establish a framework to base predication of uncatogarised samples.

However, the disadvantages of certifying these conditions would be:

(i) The difficulty of co-ordinating a uniform nationally based programme of pre-treatment of hives, feeding of colonies and monitoring to avoid mixing of early and late samples or seasonal changes or honey subjected to commercial practices or cheap foreign honey.

(ii) The siting of hives where availability of one floral species is artificially established over an area covering a radius of at least five miles from a hive. Since even a good natural heather honey contains wild clover and other species to perhaps within 10% of the total.

(iii) The monitoring and recording of sample treatment before receiving for analysis.

(iv) The modification of the amino acid analyser to be able to allow overloading to enhance the measurement of trace components, hydrolysis, identification of peptides and other compounds.

(v) The capacity to handle and analyse this sort of information would and could be conducted at a suitably equipped National research centres.

2. It would be necessary to monitor the qualitative and quantitative composition of honeys from a significant number of colonies on the same site and concurrently from up to one hundred similar sites elsewhere. The data so obtained can be used to determine the following:

(a) The composition of nectar, honeydew and pollens visited by the honeybee.

(b) The composition of secretions provided by the forager honeybee.

(c) The changes in composition of nectar deposited in the hive through to full ripened honey and subsequent capping.

(d) The variation if any in composition from cell to cell, comb to comb and of a hive at a given site. Also important is the time lag between flowering time and first and last appearance of a given nectar and pollen source in the hive as discussed by Carter (1983).

(e) A quantitative assessment of the location and floral species visited and the composition of the final honey, such as that reported by Adams, Smith and Townsend (1979).

(f) The effect of human intervention, honey extraction and blending at both amateur and professional scales. Furthermore, to establish whether the separate composition of two unifloral honeys are additive when these honeys are mixed.

(g) The effects of variation with a common source, for example, due to an apairy, seasonal changes, soil composition and the average levels of special protein fed as supplement.

(h) The effect of restricting bee food source to just one, especially sugar products and/or protein supplement.

(i) Compilation of relevant data for major and minor honey sources worldwide.

3. In addition to the above listed research it would be of use if the reduction of variable in the statistical data could be accomplished by obtaining the answers to the following questions:

(a) Does the previous years stored pollen affect and influence the ninhydrin positive substance composition of honey harvested the following year?

(b) To what degree and extent does the contribution of ninhydrin positive substances of 'Important Minor and Minor frequency class pollen' affect the overall composition of unifloral honeys?

(c) Does the feeding of pollen substitutes have, if any, significant effect on the ninhydrin positive composition of such honeys produced? If so, to what degree and extent?

(d) The identification of pollen grains to species level by conventional light microscopy using fresh and reference pollens as appropriate. Further, examination of suspect

pollen using the much higher magnification (20K) so that fine surface details essential for species identification of the *Brassica* genus can easily be accomplished. The labour of such searches can be eased and to some extend automated by using scanning image retrival. This procedure can only be done on perfect pollen and may not be successful on osmotically damaged and uncleaned pollen found in honey.

If this can be done for one country then similar analysis will be necessary for each individual honey producing country of the world. Thus, enabling the compilation of an international data bank containing information on at least a thousand plus verified reference samples per country. This would then serve not only as an indexing system, but also as a reference system for ascertaining the floral and/or geographical source of a sample by the application of this chemical technique.

In the present work the feasibility of chemical verification of floral source, especially with regard to amino acids has been accomplished. The other aims of detection of adulteration, misrepresentation have been shown to be possible and the application and future potential of computers has been investigated.

The way forward over perhaps the next ten years has been indicated but future achievements must await their turn. Upto the present, June 1987, none of these ideas have yet appeared in print.

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A P P E N D I C E S

APPENDIX I

LIST OF HONEY SAMPLES

Honey samples listed in Table IA are those obtained within the United Kingdom survey and were received over a period of 12 months commencing from October 1981 to October 1982. However, several samples were still being received upto September 1983 from members of the British Beekeepers Association. There were 192 honey samples eventually available for analysis and these were catalogued.

In Table IA:

Column one = the questionnaire number allocated to each individual beekeeper.

Column two = the approximate location of the hive as supplied by the beekeeper.

Column three = the map grid reference of the hive.

Column four = the code number allocated to each sample and serves to identify the sample throughout the analysis.

Honey samples listed in Table IB are those of foreign origin or as specified on the label. Nearly all these samples were commercially processed either at port of shipment or at the U.K. packers. There were 64 samples available for analysis and these were catalogued.

In Table IB:

Column one = the name and country of origin of a honey sample.

Column two = the name of the packer or the supplier as specified on the label or the name of the donator.

Column three = the code number allocated to each sample and serves to identify the sample throughout the analysis.

TABLE IA

LIST OF HONEY SAMPLES FOR THE UNITED KINGDOM SURVEY

Questionnaire Number	Major Lacality	Map Grid* Reference	Code Number
3b	Crawthorne	SU 8464	1
4a	Ruddington	SK 5733	2
4c	Beeley Moor	SK 2667	3
7a	Southend-on-Sea	TQ 8885	4
7b	Great Wakering	TQ 9487	5
9a	Rothbury	NU 0602	6
9b	Lesbury	NU 2412	7
9c	Longhirst	NZ 2389	8
10a	Newburn	NZ 1865	9
10b	Font	NZ 1785	10
10c	West Rainton	NZ 3247	11
11a	Galleywood (Chelmsford)	TL 7002	12
11b	Woodham Walter	TL 8006	13
12a	Bideford	SS 4526	14
12b	Bideford	SS 4526	15
13a	Hoggrills end (Coleshill)	SP 2291	16
13b	Llanfachreth	SH 7522	17
13c	Birmingham	SP 0786	18
14a	Barlaston	SJ 8938	19
14b	Barlaston	SJ 8938	20
16b	Merstham	TQ 2953	21
18a	Chalfout St. Giles	SU 9893	22
19a	Northwood	TQ 1090	23
19b	Northwood	TQ 1090	24
20a	Woodbridge	TM 2749	25
21a	Writtle	TL 6706	26
21b	Writtle	TL 6706	27
23a	Dulwich	TQ 3373	28
23b	Dulwich	TQ 3373	29
25a	Thulston	SK 4031	30
25b	Thulston	SK 4031	31
26a	Exmoor	SS 7642	32
26b	West Pennard	ST 5438	33
27a	St. Day	SW 7242	34
27b	Truro	SW 8244	35
28a	Oulton (South Leeds)	SE 3628	36
28b	Otley/Ilkely	SE 2045	37
28i	Oulton (South Leeds)	SE 3628	38

* = The grid reference given here were according to:

(1) Mason (1981).

(2) Automobile Association Great Britain Road Atlas.

Cont'd.....

Table 1A cont'd.....

Questionnaire Number	Major Lacality	Map Grid* Reference	Code Number
30a	Camberley	SU 8660	39
30b	Bagshotheath	SU 9163	40
31b	Leck Fell	SD 6779	41
32a	Wind Rush	SP 1913	42
32b	Thornbury	ST 6390	43
33a	Penrith	NY 5130	44
33b	Lazonby	NY 5439	45
34a	Brampton	NY 5361	46
34b	Brampton	NY 5361	47
35a	Pont-y-Goytre	SO 3509	48
35b	Newport	ST 3189	49
36a	Reigate	TQ 2550	50
36b	Gatwick Airport (Runway)	TQ 2841	51
37a	Egton	NZ 8106	52
38a	Helmsley Moor (Carlton)	SE 6186	53
38b	Fakenham	TF 9229	54
40a	Lydd	TR 0421	55
40b	Ashford	TR 0142	56
41a	Church Stretton	SO 4593	57
41b	Market Drayton	SJ 6734	58
42a	Rookley	SZ 5084	59
42b	Chillerton	SZ 4883	60
43a	Newbridge	SZ 4187	61
44a	Bonning Gate	SD 4895	62
44b	Stavely	SD 4798	63
45a	Hadden	NT 7836	64
45b	Morpeth	NZ 2086	65
46a	Hexham	NY 9464	66
46b	Hexham	NY 9464	67
47a	Moulton	SP 7866	68
47b	Kingsthorpe	SP 7563	69
48a	Staines	TQ 0471	70
48b	Staines	TQ 0471	71
49a	Yatton	ST 4365	72
49b	Yatton	ST 4365	73
50a	Aycliffe	NZ 2822	74
50b	Pikeston Fell	NZ 0432	75
51a	London	TQ 3079	76
52a	Bristol	ST 5872	77
52b	Weston-Super-Mare	ST 3261	78
54a	Piddington	SP 6317	79
54b	Long Creadon	SP 6908	80
56a	Jarrow	NZ 3265	81

Cont'd.....

Table IA cont'd.....

Questionnaire Number	Major Lacality	Map Grid* Reference	Code Number
56b	Woolsingham	NZ 1969	82
57a	Starcross	SX 9781	83
57b	Starcross	SX 9781	84
58a	Bolton	SD 7109	85
58b	Bolton	SD 7109	86
60a	West Hanney	SU 4092	87
60b	Wantage	SU 4087	88
61a	Cobham	TQ 1060	89
61b	Cobham	TQ 1060	90
61c	Cobham	TQ 1060	91
62a	Sawdon	SE 9485	92
62b	Bickley	SE 9192	93
63a	Goring	SU 6080	94
63b	Whitchurch Hill	SU 6479	95
64a	Crowthorn	SU 8464	96
64b	Crowthorn	SU 8464	97
65a	Newton Tracey	SS 5226	98
65b	East Combe	SS 8904	99
68a	Huntshaw	SS 5023	100
68b	Torrington	SS 4919	101
69a	Ottery St. Mary	SY 0995	102
69b	Ottery St. Mary	SY 0995	103
70a	Hoddesdon	TL 3709	104
70b	Hoddesdon	TL 3709	105
71a	Cheshunt	TL 3502	106
71b	Waitham Cross	TL 3600	107
72a	Barham	TL 1375	108
72b	Barham	TL 1375	109
73a	Houghton Hill	TL 2972	110
73b	Houghton Hill	TL 2972	111
75a	Plaistow	TQ 0030	112
75b	Billinghurst	TQ 0825	113
76a	Stevenage	TL 2325	114
76b	Stevenage	TL 2325	115
77a	Craven Arms	SO 4382	116
77b	Craven Arms	SO 4382	117
78a	South Cerney	SU 0497	118
78b	South Cerney	SU 0497	119
79a	Findern	SK 3030	120
79b	Littleover	SK 3234	121
80a	Pinner	TQ 1289	122
80b	Pinner	TQ 1289	123

Cont'd.....

Table IA cont'd.....

Questionnaire Number	Major Locality	Map Grid* Reference	Code Number
81a	Gt. Dunmow	TL 6221	124
85a	Broadstairs	TK 3967	125
85b	Broadstairs	TK 3967	126
86a	Lewes	TQ 4110	127
86b	Lewes	TQ 4110	128
87a	Birchington	TR 3069	129
87b	Birchington	TR 3069	130
88b	Broxted	TL 5727	131
89a	Broxted	TL 5727	132
91a	Rochford	SO 6268	133
91b	Rochford	SO 6268	134
93a	Stainton-Le-Vale	TF 1794	135
93b	Moortown	TF 0699	136
94a	Appleby	SE 9514	137
98a	Bury St. Edmonds	TL 8564	138
98b	Bradfield St. George	TL 9059	139
99a	Hale	ST 4682	140
100a	Lowington	SP 2069	141
100b	Barston	SP 2078	142
101a	Toton	SK 5034	143
101b	Beeston	SK 5336	144
102a	Bradmore	SK 5831	145
102b	Nottingham	SK 5640	146
103R1	Ollerton	SJ 7776	147
103R2	Ollerton	SJ 7776	148
103R3	Ollerton	SJ 7776	149
103R4	Ollerton	SJ 7776	150
103R5	Ollerton	SJ 7776	151
103R6	Ollerton	SJ 7776	152
103R7	Ollerton	SJ 7776	153
103R8	Ollerton	SJ 7776	154
103R9	Ollerton	SJ 7776	155
105a	Leyland	SD 5421	156
105b	Waddington	SD 7243	157
105d	Preesall	SD 3646	158
105e	Bolton	SD 7109	159
105f	Mellor Brook	SD 6331	160
105g	Cottam	SD 4932	161
105h	Southport	SD 3316	162
105j	Dalton	SD 4907	163
105K	Bretherton	SD 4720	164
107a	Hoar Cross	SK 1223	165
107b	Hoar Cross	SK 1223	166

Cont'd.....

Table IA cont'd.....

Questionnaire Number	Major Locality	Map Grid* Reference	Code Number
108a	Hewelsfield	SO 5602	167
108b	Hewelsfield	SO 5602	168
108c	Srinagar	Kashmir (India)	169
109a	Denham	TQ 0386	170
109b	Stanmore	TQ 1692	171
110a	Halse	ST 1327	172
110b	Gharlton Mackrell	ST 5228	173
111a	Wiggaton	SY 1093	174
112a	Feering	TL 8720	175
112b	Feering	TL 8720	176
114a	Tunbridge Wells	TQ 5839	177
114b	Bodiam	TQ 7826	178
114c	Comberton	TL 3856	179
114d	Comberton	TL 3856	180
114f	Trowbridge	ST 8557	181
114g	Trowbridge	ST 8557	182
114k	West Calder	NT 0613	183
1141	West Calder	NT 0613	184
115a	Shrewbury	SJ 4912	185
115b	Shrewbury	SJ 4912	186
115c	Unknown	-	187
117a	New Mill	SP 9212	188
117b	Hemel Hempstead	TL 0506	189
118	Clay Cross	SK 3963	190
119	Mylor Bridge	SW 8036	191
122	Unknown	-	192

TABLE IB

LIST OF THE FOREIGN AND COMMERCIAL HONEY SAMPLES

Name and Country of origin	Name of packer or supplier or donator	Code Number
Austria	Marchtrenk, Austria	193
Australia* Banksia	donated by Manley Ratcliffe Ltd., Berinsfield, Oxford (1)	194
Australia Bluebell	Crabtree and Evelyn, London	195
Australia Bluebell (H470)	donated by Mr. Rex Sawyer (2)	196
Australia	Rowse Honey Ltd., Ewelme, Oxford	197
Argentina Clover	" "	198
Canada*	donated by (1)	199
Canada Clover	Rowse Honey Ltd., Ewelme, Oxford	200
Canada Clover (H666)	donated by (2)	201
Canada Sunflower (H669)	" "	202
Canada British Colombia (H707)	" "	203
Canada Clover Samantha	D. J. Sales Ltd., Salford	204
Canada Clover Pure Sainsbury's	J. Sainsbury Ltd., London	205
Canada Clover Pure Tiptree's	Wilkin and Sons Ltd., Tiptree	206
Cayman Island Coconut	donated by Mr. Harrison of Ashford	207
China Acacia*	donated by (1)	208
China Buckwheat*	" "	209
China Eucalyptus* Light Amber	" "	210
China Light* Amber	" "	211
China White*	" "	212
China White	donated by a Beekeeper of Middlesex	213
Chile*	donated by (1)	214
France F1	purchased on site, La Belliole, France, by Mr. J. M. Carter	215
France F2	" " " "	216
France F3	" " " "	217
France F4	purchased on site, Charente, France, by Mr. J. M. Carter	218
France F5	" " " "	219

* = These are bulk honeys in steel drums as shipped by importers, origin and information as stated on consignment.

1 = Manley Ratcliffe Ltd., Berinsfield, Oxford.

2 = Mr. Rex Sawyer.

Cont'd.....

Table IB cont'd.....

Name and Country of origin	Name and packer or supplier or donator	Code Number
Greece Nectar	Halkidiki, Thasos, North Greece	220
Greece Nektar (H689)	donated by (2)	221
Hungary* Polyflora	donated by (1)	222
India Charak	Charak Pharmaceuticals (India) PVT Ltd., India	223
India Gujarat (1)	purchased on site, by Mr. P. M. Mistry	224
India Gujarat (2)	" " " "	225
Malta	purchased on site, by Mr. C. Airey	226
Mexico* Yucatan	donated by (1)	227
Mexico Pure Sainsbury's	J. Sainsbury Ltd., London	228
New Zealand*	donated by (1)	229
New Zealand Clover	Manley Ratcliffe Ltd., Oxford	230
Russia White	Parish and Fenn Ltd., Surrey	231
Spain Orange Blossom	The Honeycomb Co., ELLEL. R. P. Lancaster	232
Spain Sunflower	Cotswolds Windrush Honey Farms Ltd., Witney	233
Gales Honey	Colman's of Norwich, Carrow, Norwich	234
Gales Honey (A)	" " " "	235
Gales Honey (B)	" " " "	236
Sainsbury's Pure Acacia	J. Sainsbury Ltd., London	237
Sainsbury's Pure Clear	" "	238
Sainsbury's Pure Set	" "	239
Rowse's Cut Comb (1)	Rowse Honey Ltd., Ewelme, Oxford	240
Rowse's Cut Comb (2)	" " "	241
Ratcliffe's Pure Clover	Manley Ratcliffe Ltd., Oxford	242
MR68	donated by (1)	243
MR67	"	244
MR66	"	245
Yellow Box*	"	246
Comb Honey	supplied by Dr. Croft	247
English Pure	Rowse Honey Ltd., Ewelme, Oxford	248
English Pure Staffordshire	Peirson Shaffalong, Cheddleton	249
English Pure	Dr. Croft's Apairy, Chorley, Lancashire	250
English Pennine Heather	Pennine Bee Farms, Galgate, Nr. Lancaster	251
English	National Honey Show, Caxton Hall, Westminister, London 1979	252
English Pure M8418	donated by Preston Public Aalyst's Laboratory	253
English Clover M8522	" " " "	254
Man Made Honey	supplied by Dr. Croft	255
Sainsbury's Golden Syrup	J. Sainsbury Ltd., London	256
Lancashire Tarleton	Reference honey, Siddiqui (1981)	257
Linconshire	" " "	258
Northern Ireland	" " "	259
Pure Honey	" " "	260
English Chorley	Dr. Croft's Apairy, Chorley, Lancashire	261

APPENDIX II

QUESTIONNAIRE

Given below is the list of the questions on the questionnaires which were sent to the beekeepers who took part in the United Kingdom survey. For example:

(1) Name .....

Address .....

.....

(2) Approximate location of the colony:

Situated on 'outskirts' of a farm.....

fruit, beans etc.....

Map Grid Reference .....

(3) Date of removal of honey from comb .?./Jul/81

(4) Estimated main floral source: Beans (stick) softfruit

(5) Has the honey been heated? XXX/No

If yes, please give conditions: .....

(6) Has this colony been fed sugar? XXX/No

If yes, please state the quantity, and form, given during previous 12 months. .....

(7) Yield from colony during previous 5 months: For this analysis unable to judge but averaged 30lbs from 10 colonies.

(8) Any other comments: .....

.....

Note: The name and address of the participant beekeeper and the questionnaire number have not been quoted so as to retain confidentiality. The responses to the question number are according to those quoted by the beekeeper.

APPENDIX III

DETERMINATION OF THE AMINO ACID CONCENTRATION IN A HONEY SAMPLE

A honey sample after concentration, as described, was mixed with an equal volume (25 µl) of buffer containing norleucine and duplicate (20 µl) portions were applied; one to each of the Technicon resin columns. The absorbance of the reaction mixture and eluate was determined at 570 nm for amino acid and 440 nm for proline. The data obtained was observed on a chart paper as a trace response curve whose peak shape approximated to that of a triangle. The normal definition of a triangle, that is, half base multiplied by the perpendicular height could not be applied because of the varying position of the curve limits. Hence, an equivalent definition which has been used universally, as curve peak height multiplied by the width at half curve peak height was applied. The area under each curve peak, thus obtained, was proportional to the amino acid concentration, that is

$$\text{area} = k[\text{amino acid concentration}] \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 1}$$

where k is a constant determined by pre-calibrating the instrument with authentic standard solutions containing 25 nMoles of each amino acid. Due to variations in local heating, pump tubings and measurements of the colour reaction at a possible non-equilibrium position, it was essential to include in each sample a constant amount of internal standard to correct for these variations. The standard usually chosen norleucine eluted near the middle of the eluted amino acid range although other standards could be used for special purposes. The order of elution of the standard eighteen amino acids and ammonia were: lysine, histidine, ammonia, arginine, aspartic acid, thrionine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, norleucine, tyrosine and phenylalanine.

However, due to the technique of preparing the honey amino acid concentrates as described in the methods section, the ammonia peak was very high and also that there were naturally very low levels of the amino acids histidine, arginine, cysteine and methionine and significant peaks due to these amino acids were rarely observed. Therefore, only fourteen amino acids were commonly recorded in the honey samples.

The concentration of an amino acid in a concentrated honey amino acid sample was evaluated from the area under the trace responce curve using the equation derived below.

Derivation of Equation

The relationship between the concentration of an absorbing substance and the measured value of the absorbed radiation and the fact that a given sample always absorbs the same proportion of the incident radiation regardless of its intensity, is defined by the Beer-Lambert's Law. This Law can be expressed mathematically as:

$$A = \varepsilon c l \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 2}$$

where A = absorbance of radiation

ε = molar absorption coefficient of a substance (1 mol^{-1} cm^{-1})

c = concentration of a substance (mol l^{-1})

l = light path (cm).

When comparing the absorbance value for a particular amino acid present in both the standard solution and the concentrated honey amino acid sample the molar absorption coefficient, constant path length and the molecular weight can be regarded as constant for measurements at the same wavelength.

Thus: $A_s = \varepsilon_s c_s l_s$ for the standard amino acid solution.

$A_H = \varepsilon_H c_H l_H$ for the concentrated honey amino acid solution.

Hence:

$$\frac{A_s}{A_H} = \frac{\varepsilon_s\ c_s\ l_s}{\varepsilon_H\ c_H\ l_H} = \frac{c_s}{c_H} \qquad \ldots\ldots \text{Equation 3}$$

By using an internal standard norleucine of constant concentration in both the standard and honey amino acid solutions the ratio A_{SN}/A_{HN} derived similarly as in equation 3, can be used as a multiplication factor to compensate for changes in instrumentation parameters. Thus:

$$\frac{A_s}{A_H} = \frac{C_s \times A_{SN}}{C_H \times A_{HN}} \qquad \ldots\ldots \text{Equation 4}$$

Rearranging equation 4 the concentration of an amino acid in the concentrated honey amino acid - internal standard norleucine mixture (20 µl) applied to the analyser column can be obtained. Thus:

$$CH = \frac{C_S \times A_{SN} \times A_H}{A_{HN} \times A_S} \qquad \ldots\ldots \text{Equation 5}$$

The ratio A_{SN}/A_S is the norleucine equivalent of the corresponding amino acid response curve from the analysis of the standard amino acid to that of the concentrated honey amino acid sample and was denoted by the term NESP, and C_S has the value 25 nMoles, that is the concentration of each amino acid in the standard amino acid solution. Hence, equation 5 can be written as:

$$C_H = \frac{C_S \times NESP \times 25\ (nMoles)}{A_{HN}} \qquad \ldots\ldots \text{Equation 5a}$$

The value of C_H in the 25 µl of the original concentrated honey amino acid solution prior to mixing with the internal standard norleucine (25 µl) can be obtained by multiplying equation 5a by the factor 50/20. Thus:

$$C_H = \frac{A_H \times NESP \times 25\ (nMoles)}{A_{HN}} \times \frac{50\ (\mu l)}{20\ (\mu l)} \qquad \ldots\ldots \text{Equation 6}$$

The amount of amino acid present in 10.0g of honey can be obtained by multiplying equation 6 by the factor $\frac{PH}{25}$, where PH is the total weight of 10% (v/v) propan-2-ol/0.5M HCl solution (1.5 mls) plus the amount of the disssolved amino acid concentrated from honey (10.0g). It was found at least approximately that 1 ml volume of this concentrated honey amino acid solution was equivalent to 1 gram in weight and the total weight of the concentrated honey amino acid solution (PH) was expressed in volumetric units, microlitres (µl). Thus:

$$C_H = \frac{A_H \times NESP \times 25\ (nMoles)}{A_{HN}} \times \frac{50\ (\mu l)}{20\ (\mu l)} \times \frac{PH\ (\mu l)}{25\ (\mu l)} \qquad \ldots\ldots \text{Equation 7}$$

Moreover, the concentration measurements were expressed as nMoles per gram of honey. Therefore, equation 7 was divided by the factor 10.0. Thus:

$$C_H = \frac{A_H \times NESP \times 25\ (nMoles)}{A_{HN}} \times \frac{50\ \mu l}{20\ \mu l} \times \frac{PH\ (\mu l)}{25\ (\mu l)} \div 10.0$$

$$= \text{nMoles per gram of honey} \qquad \ldots\ldots \text{Equation 8}$$

N.B. It was noted from the trace response curve chart paper that when two or more trace curves occurred close by or overlapped to some extent the curve limits for each trace curve did not always occur on the baseline. Therefore, in these rare cases approximations were devised to determine the height and width at half height of curve peak. The approximations procedure was than utilized for similar occurring trace response curves throughout the calculations of amino acid concentrations.

A Typical Example of Calculation

The concentration of aspartic acid in the U.K. survey sample Code No. 27 was calculated using equation 8 as follows:

$$C_H = \frac{A_H \times NESP \times 25\ (nMoles) \times 50\ (\mu l) \times PH\ (\mu l)}{A_{HN} \times 20\ (\mu l) \times 25\ (\mu l) \times 10.0(g)}$$

where C_H = Concentration of aspartic acid (nMoles per g of honey).

A_H = Area under the trace response curve for aspartic acid in U.K. Sample 27.

= 2.0 cm (height) x 0.4 cm (width ½ height) = 0.8 cm^2.

$NESP = \frac{A_{SN}}{A_S} = \frac{\text{Area under the curve for norleucine}}{\text{Area under the curve for aspartic acid}}$ for the standard amino acid solution

$= \frac{14.6\ cm\ (h) \times 0.4\ cm\ (w\frac{1}{2}h)}{12.5\ cm\ (h) \times 0.4\ cm\ (w\frac{1}{2}h)} = 1.17$

PH = 1530 µl (1.53g)

A_{HN} = Area under the trace response curve for internal standard norleucine applied to U.K. sample 27.

= 13.7 cm (h) x 0.4 cm ($w\frac{1}{2}h$)

= 5.48 cm^2.

Therefore:

$$C_H = \frac{0.8\ (cm^2) \times 1.17 \times 25\ (nMoles) \times 50\ (\mu l) \times 1530\ (\mu l)}{5.48\ (cm^2) \times 20\ (\mu l) \times 25\ (\mu l) \times 10.0(g)}$$

= 65.33 nMoles of aspartic acid in 1g of U.K. sample Code No. 27.

APPENDIX IV

COMPUTATION

Described below is the format of the data arranged in the U.K. survey and the foreign and commercial data files. This format is as follows:

The concentration of the free amino acids used were those calculated from the area under the trace response curves on the chart paper output as detected by the amino acid analyser. There were seventeen amino acids detected and of these only thirteen whose curve peaks could be adequately measured were considered. These thirteen amino acids were:

lysine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine and phenylalanine

in that order.

The questionnaire as sent to the beekeepers contained eight questions of which the stated responses to question numbers, 3, 5 and 6 were used here. The relevant questions were: 'Date of removal of honey from comb'?, question 3; 'Has the honey been heated'? Yes or No, question 5; and 'Has this colony been fed sugar'? Yes or No, question 6.

The classification of the distribution of the pollen grains into frequency classes for each sample of honey was used. Out of the four pollen classes only the predominant and the secondary pollan classes were considered. Honey samples containing a given pollen which occurred over 45% of the total pollen counted were allocated into the predominant pollen class. Those honey samples whose pollen content ranged between 16% and 45% were allocated into the secondary pollen class. Furthermore, in this case for honey samples which occurred in the secondary pollen class; only the first two pollens in decending order of percentage were considered. All the two hundred and fifty six samples of honey contained either the predominant or the secondary pollens.

The pollen content, that is, the amount of pollen grains present on the prepared microscope pollen slides was determined quantitatively by visual microscopical examination. Values on an arbitrary scale of one to five were selected, where one represented the lowest or zero pollen content and five represented the highest pollen content. The intermediate numbers 2, 3 and 4 represented the intermediate pollen contents of low, medium and high, respectively.

The occasional presence of fifteen other amino acids besides the thirteen amino acids which were quantitatively estimated by the amino acid analyser and common to all the samples were also detected by the technique of paper electrophoresis and chromatography. Besides these twenty eight amino acids eleven other ninhydrin complex colour spots whose identity could not be ascertained were also occasionally found to be present. These fifteen amino acids and the eleven unidentified ninhydrin complex colour spots thus detected were used for qualitative analysis on the computer. The identity of the thirteen amino acids has been given above. The other fifteen amino acids were identified to be: α- and γ- amino butyric acid, arginine, asparagine, cysteine, glucosamine, glutamine, histidine, methionine, tryptophan, hydroxyproline, hydroxypipecolic acid, methyl-histidine, β-alanine and pipecolic acid. The unidentified twelve ninhydrin complex spots were labelled as p, X, ξ, δ, ζ, ε, π, ω, λ, θ, σ and Ω.

All this data were compiled in the order listed above into a data file for each individual honey sample.

There were two data files compiled, one contained the United Kingdom survey while the other contained the foreign and commercial honeys.

In each of these two data files, for each sample of honey the data mentioned above were arranged into four lines. Each line composed of eighty characters, one character representing a column. The first two lines of the four lines contained the concentration values of the thirteen free amino acids quoted to two decimal places. These two lines were divided into sixteen columns, eight columns per line. Each column composed of ten characters. The first line contained the concentration values of the first eight amino acids of the thirteen. These eight amino acids were: lysine, aspartic acid, threonine, serine, glutamic acid, proline, glycine and alanine, in that order. The second line contained the concentration values of the remaining five amino acids valine, isoleucine, leucine, tyrosine and phenylalanine, in that order. Line three of the four lines was divided into thirteen columns of five

characters each except for column twelve which was of one character. This third line contained the answers to questionnaire questions 3, 5 and 6. This was followed by the predominant pollen and its percentage value and then by the first secondary pollen and its percentage value. This was then followed by the second secondary pollen and its percentage value. This was in turn followed by the unidentified and shrivelled pollen percentage values, quoted to one decimal place and then finally by the pollen content. The last line contained the information on the presence of the fifteen amino acids and the two unidentified ninhydrin complex spots. This fourth line was divided into seventeen columns of three characters each. These fifteen amino acids were listed in the following order: α-, and γ-aminobutyric acid, arginine, asparagine, cysteine, glucosamine, glutamine, histidine, methionine, tryptophan, hydroxyproline, hydroxypipecolic acid, methyl-histidine, β-alanine, pipecolic acid and the unidentified spots 'p' and 'x'.

Moreover, in the second data file which contained all the data for the sixty four foreign and commercial honey samples the data were arranged in a similar manner to that of the United Kingdom survey data file. The only difference in this second data file was that there were no questionnaire quetions 3, 5 and 6 apart from these questionnaire questions all the other data were exactly the same as those in the United Kingdom survey file. These questionnaire questions were replaced by information regarding the country of origin and the commercial processing of the honey samples. Hence, question 3 was replaced by the information regarding the country of origin for each sample. The column representing the question 5 was left blank. Question 6 was replaced by the following question: 'Has this sample of honey been commercially processed'? Yes or No.

It is generally required by the SPSS to code the data. The code can take the form of words, letters or numbers. The words or letters can often complicate an SPSS command and also some procedures in the SPSS require numerical data values. Hence, a numerical coding scheme was adopted. The numerical codes used in this case are given in the control statement 'VALUE LABEL' shown in Appendix V (b). The numerical codes used for identifying pollen grains are given in Table 30. Actual numbers such as the amino acid concentration values and the pollen percentage values did not require coding.

APPENDIX V

A DISCRIMINANT ANALYSIS

Described below is the order in which the DISCRIMINANT analysis on the one hundred and ninety two samples of the U.K. survey honeys, the sixty four samples of the foreign and commercial honeys and also on both the combined honeys was performed using the SPSS.

(a) United Kingdom Survey Honey Samples

The results of the microscopic examination and evaluation of the distribution of pollens in each of the samples of the U.K. survey showed that there were nine major pollens which occurred in the predominant frequency class. When specified the SPSS allocated one hundred and four samples of honey which were classified into one of the pollen groups specified. The remaining samples were not dealt with because these had either missing or out of range amino acid concentration values, or they were composed of secondary or multiple pollen.

In order to improve the percentage of correct classification of the honey samples in each of the pollen groups, a number of factors were considered. These factors were: a transformation of the original amino acid concentration values, reducing the number of pollen groups and to minimise the effects of the presence of other pollens besides the predominant pollen which may cause overlapping between pollen groups.

Two transformations of the original amino acid concentration values were chosen. These transformations were the natural logarithms and the square root. The square root transformation improved the discrimination better than the natural logarithms. This square root transformation makes the variance-covariance matrix of the amino acid concentration data vector more nearly equal across the pollen groups. Therefore, the square root transformation was chosen and adhered to throughout the following analyses.

The four pollen groups, *Aesculus*, *Impatiens*, *Vicia* and *Clemantes*, contained only five samples of honey between them. This number of honey samples for the four pollen groups were considered to be inadequate to produce a valid discriminant analysis. Therefore, these four pollen groups were not included in any of the following analysis.

Further, in the predominant pollen frequency class there were a number of honey samples which contained both the predominant pollen as well as the secondary pollen. These samples of honey were found to occur in the 45% to the 60% region of the predominant pollen frequency class. Discriminant analysis was performed to check whether the presence of these secondary pollen or the presence of the other pollens in the important minor pollen frequency class were affecting the discrimination between the pollen groups. Thus, the predominant pollen class was further divided into two subclasses. These two subclasses were labelled as the unifloral and the multifloral pollen subclasses. Preliminary tests using the discriminant analysis was carried out on four pollen groups to discriminate between the unifloral and the multifloral pollen subclasses. The four pollen groups were: *Brassica*, *Trifolium repens*, *Castanea* and *Calluna*. The *Myosotis* pollen group was considered as the unifloral pollen subclass since the amount of this pollen present in each of these honey samples was over 95%.

In all six preliminary tests were carried out and in each of these tests the unifloral pollen subclass group was defined as those honey samples containing pollen over 60%, 70%, 75%, 80% and 85%. The multifloral pollen subclass group was defined as those honey samples containing pollen present between 45% and 60%, 45% and 65%, 45% and 70%, 45% and 75%, 45% and 80% and 45% and 85%, respectively. The results showed that there was considerable overlap in each of the six tests between the unifloral and multifloral subclass pollen groups.

In order to minimise this overlap between the unifloral and multifloral pollen groups, the multifloral pollen group was defined as those honey samples containing pollen present between 45% and 60% only. The unifloral pollen group was defined as those honey samples containing pollen present over 70%. Introduction of this 10% gap between the two pollen subclasses reduced to a certain extent the overlap effect of the honey samples containing pollen present between 60% and 65%, 65% and 70%.

Further tests were carried out using the discriminant analysis to eliminate the overlap between the unifloral and multifloral pollen subclasses in each of the four pollen groups. In these tests the multifloral subclass pollen group was defined as mentioned before. The unifloral subclass pollen group were defined as those honey samples containing pollen present over 75%, 80% and 85%. The results showed that as the gap between the multifloral and the unifloral subclass pollen groups was increased, the overlap between these two pollen groups was eliminated. Typically, Figure 27 (results) the histogram obtained for the *Brassica* pollen group show the elimination of overlap between the two pollen groups.

These results also showed that as the percentage of the pollen present in the unifloral subclass pollen group was increased to eliminate overlapping the number of samples in each of the four pollen groups decreased from forty six at 70% pollen to twenty seven at 80% pollen. Therefore, it was considered that when discriminating between the five pollen groups honey samples which contain pollen present over 70% would be considered as unifloral honeys. This 70% limit was chosen because each of the five pollen groups contained adequate number of honey samples which would give valid discriminant analysis. The SPSS control file described and detailed in Appendix VI (c) was used to initiate the SPSS as mentioned previously. In this analysis, the honey samples in each of the pollen groups were divided into two subsets. One subset, was used for calculating the Fisher's linear discriminant functions and the other for estimating the misclassification rate, however, all the samples were classified. This dividing of the honey samples into two subsets was also used in all the following analyses.

In the United Kingdom, honey from the *Brassica*, *Trifolium repens* and *Calluna* plants are the major sources of honey. The honey from these three plants are of considerable importance to the beekeepers of the United Kingdom. Discriminant analysis was performed on the honey samples representing each of these three pollen groups. The SPSS control file detailed in Appendix VI (c) was used to initiate the SPSS. In this analysis, the *Castanea* and *Myosotis* pollen groups were ommitted from analysis, in that, control statements 58 and 60 were deleted from the SPSS control file.

From the response received to questionnaire question 3 which requested the 'Date of removal of honey from the comb' it was observed that nearly all the honey samples were either harvested in the crop year 1981 or 1982. Discriminant analysis was performed to check whether there were any significant differences in the amino acid composition of the honeys produced during these two crop years. The SPSS control file detailed in Appendix VI (c) was used to initiate the SPSS as mentioned previously. In this analysis, the control statements 56 to 60 were replaced by those given in Appendix VI (d)(i).

Similar discriminant analysis to that of above was also performed on honey samples representatives of the individual pollen groups which were harvested in the crop years 1981 and 1982. The five pollen groups chosen in the previous analysis were used. The control statements 56 to 60 of Appendix VI (c) were replaced by those given in part (d)(ii)1-5. Statistical results were obtained for only three of these pollen groups. These three pollen groups were *Brassica*, *Trifolium repens* and *Castanea*. For the other two pollen groups there were not enough samples of honey in either of the years specified and hence, statistical analysis for these two pollen groups could not be computed.

From the response received to questionnaire question 5 which requested whether the honey had been heated or not. Discriminant analysis was performed to test whether heating a sample of honey effects its amino acid compositions. The SPSS control file detailed in Appendix VI (c) was used to initiate the SPSS. In this analysis, the control statements 56 to 60 were replaced by those given in Appendix VI (e)(i). Similar discriminant analysis to that of above were performed on honey samples representative of the individual pollen groups which were either heated or not heated. The five pollen groups chosen in the previous analysis were used. Statistical results were obtained for only three of these pollen groups. These three pollen groups were: *Brassica*, *Castanea* and *Myosotis*. For the other two pollen groups there were not enough samples of honey in either of the 'heat' groups and hence, statistical analysis for those two pollen groups could not be computed.

From the response received to questionnaire question 6 which requested whether the colony had been fed sugar. Discriminant analysis was performed to test whether feeding sugar to a colony of bees during autumn, winter and spring effect the overall amino acid composition of honey from that colony. The SPSS control file detailed in Appendix VI (c) was used to initiate the SPSS. In this analysis, again the control statements 56 to 60 were replaced by those given in Appendix VI (e) (ii).

Similar discriminant analysis to that of above was performed on honey samples representatives of individual pollen groups which were collected from colonies that had been fed sugar or otherwise. The five pollen groups chosen in the previous analysis were used. Statistical results were obtained for all five pollen groups.

In order to assess whether the honey stores from colonies that had been fed sugar in the autumn months are stored and subsequently harvested in the New Year's crop. Those samples which were autumn fed were regarded as samples which had not been fed sugar. Discriminant analysis as mentioned previously for the autumn, winter and spring fed was carried out on the winter and spring fed samples. The SPSS control file detailed in Appendix VI (C) was used with the modified control statements 56 to 60.

Similar discriminant analysis to that of above was also performed on honey samples representatives of the individual pollen groups which were collected from colonies that had been either sugar fed or otherwise. The five pollen groups considered in the previous analysis were chosen. Statistical results for the *Calluna* pollen group could not be computed due to the lack of honey samples in either of the 'SUGAR' groups.

Furthermore, verification of sugar feeding to colonies of bees was determined by testing the relative concentration of the amino acid proline to that of the other amino acids. The honey samples used in this case were the autumn, winter and spring fed. The SPSS control file detailed in Appendix VI (c) was used to initiate the SPSS. In this case, the control statements 56 to 60 were replaced by those given in Appendix VI (e)(ii). Since the results did not show any improvements in this classification analysis. The analysis of the five pollen groups was not performed.

From the responses of questionnaire questions 5 and 6, it was observed that some of the honey samples were both heated and from colonies that had been fed sugar and visa versa. There are four possible combinations of these two questionnaire questions differentiated into two groups. These four combinations are given in Appendix VI (f). Discriminant analysis was performed on each of these four combinations. The SPSS control file detailed in Appendix VI (c) was used. In these analysis, the control statements 56 to 60 were replaced by one of the four combination statements given in Appendix VI (f).

Similar discriminant analysis was performed to that of above on honey samples representatives of the individual pollen groups for each of the four combinations.

(b) Foreign and Commercial Honey Samples

Discriminant analysis was performed on the samples of honey originating from Australia, Canada, Mexico, New Zealand and the English commercial samples. The SPSS control file detailed and described in Appendix VII (a) was used to initiate the SPSS.

In the next analysis, the English commercial samples were omitted. Discriminant analysis was performed on the four foreign country group samples, Australia, Canada, Mexico and New Zealand. The SPSS control file detailed in Appendix VII (a) was used. The control statements 54 to 58 were replaced by those given in Appendix VII (b)(i). Further, the samples of honey from China were also included in the above analysis. Discriminant analysis was performed as mentioned previously. The SPSS control file detailed in Appendix VII (a) was used. The control statements 54 to 58 were replaced by those given in Appendix VII (b)(ii).

From the foreign and commercial honey samples that were available. Discriminant analysis was performed on honey samples from six selected countries. These countries were the same as those mentioned in the previous analysis except that the France honey samples were also included. The control statements 54 to 58 of the SPSS control file detailed in Appendix VII (a) were replaced by those given in Appendix VII (b)(iii).

In the next analysis the Mexico and New Zealand honey samples were replaced by those samples originating from India. The control statements 54 to 58 of the SPSS control file in Appendix VII (a) were replaced by those given in Appendix VII (iv).

From the microscopical examination and evaluation it was noticed that the pollen contents of the English, France and Spain honey samples showed considerable similarities. Hence, discriminant analysis was carried out to test whether there are any differences in the amino acid composition between the English and European honeys. The control statements 54 to 58 of the SPSS control file in Appendix VII (a) were replaced by those given in Appendix VII (b)(v).

Also it was noticed during the microscopical examination that the Austria and Greece honey samples contained algael cells and other unidentifiable microscopical elements. It was considered that these samples may be of honeydew origin. Thus, discriminant analysis was performed on honey samples of European origin, that is, Austria, France, Greece and Spain. Further the honey samples of Austria and Greece were considered to originate from honeydew and hence the code for Greece was changed to that of Austria. The honey samples of France and Spain was considered to originate from nectar and hence, the code for Spain was changed to that of France. The control statements 54 to 58 in the SPSS control file detailed in Appendix VII (a) were replaced by those given in Appendix VII (b)(vi).

It was also noticed during the microscopical examination that the pollen grains of *Trifolium repens* and *Melilotus* had very similar features. It was also noted that these two pollen grains belonged to the sample plant family, *Leguminosae*.. Discriminant analysis was performed to test whether there are any differences in the amino acid composition of these honeys. The control statements 54 to 58 of the SPSS control file detailed in Appendix VII (a) were replaced by those given in Appendix VII (b)(vii). Further, it was also noticed that the pollen grain of *Lotus* also belonged to the *Leguminosae* plant family. The honey samples containing this pollen were also included in the previous analysis. Refer to Appendix VII (b)(viii) for the control statements used.

Since there were fourteen samples in the foreign and commercial honeys whose country of origin was not known, it was thought that on the basis of their pollen content these samples be allocated the code for the country which they were most likely to have originated from. Thus, sample numbers 237, 238, 239 and 242 were allocated the code for Australia; the sample numbers 240, 241, 243, 244 and 246 were allocated the code for Mexico and the sample number 247 was allocated the code for New Zealand. Further, sample number 205 was predicted by the SPSS in a previous analysis to have originated from Australia and was allocated the appropriate code. Similarly, sample number 231 whose pollen content was similar to that of the Mexico samples was also allocated the appropriate code. Also, the sample number 253 whose authenticity was suspect and from its pollen content it was attributed the code for Australia. The sample number 234, 235 and 236 from their amino acid maps were thought to have originated from Australia and were allocated the appropriate code. The SPSS control file given in Appendix VII (a) with the replaced control statements 54 to 58 by those listed in Appendix VII (b)(i) was used.

Some of the samples received had been commercially processed and bottled, while others were not. Discriminant analysis was performed using the SPSS control file detailed in Appendix VII (a). The control statements 54 to 58 were replaced by those listed in Appedix VII (b)(ix).

(c) Combined Honey Samples of the United Kingdom Survey and the Foreign and Commercial

Discriminant analysis was performed on the two hundred and fifty six honey samples to test whether there were any differences in the amino acid composition of the United Kingdom and foreign and commercial samples. Hence, in this analysis, the five 'IF' control statements 56 to 60 of Appendix VII (a) were replaced by those given in Appendix VII (c)(i). The SPSS was initiated as mentioned before.

Discriminant analysis was performed to discriminate between the U.K. survey, Australia, Canada, China, France, India and England samples. The control statements given in Appendix VII (c)(ii) were used.

From the evaluation of the distribution of the pollen grains it was observed that both the United Kingdom survey and the foreign and commercial honey samples contained the *Brassica*, *Trifolium repens* and *Castanea* pollen in the unifloral subclass group. Discriminant analysis was performed using the SPSS control file detailed in Appendix VII (a). In this case, the control statements 59 to 60 were omitted.

APPENDIX VI

DISCRIMINANT ANALYSIS OF THE U.K. SURVEY HONEYS

(a) Definition of Discrimination and Mahalanobis Distance

Discrimination, in this case, for example, is between the pollen groups. A sample of honey can be allocated to one of a number of groups or population according to the pollen it contains. For each honey sample the concentration of the thirteen free amino acids were measured. It is on the basis of these thirteen measurements that a honey sample of unknown floral origin is allocated to a pollen group.

For each honey sample the thirteen free amino acid measurements can be thought of as co-ordinates in a thirteen dimensional space. Thus, in one pollen group the honey samples from that particular pollen group will form a cluster of points. The problem is to define a boundary in this space to separate the different groups. Once the boundaries have been chosen a honey sample of unknown floral origin can then be allocated to a pollen group. Since there will be no true boundaries (due to randomness in the measurements), that is, the populations 'over lap' in this space, errors will sometimes be made when allocating a honey sample to a group. The statistical methods of 'Discriminant analysis' have been developed to select boundaries which will minimise the probability of making these errors. One such procedure is to use the Fisher's discriminant function. This function is obtained by choosing a linear combination of the amino acid concentrations which defines the direction of maximal group separation in the sense that the means of the projections of the honey samples from the pollen groups are maximally apart (relative to the variance of the projections around their respective means). A number of linear combinations are known as discriminant co-ordinates or canonical variates. These give varying degrees of separation. Usually the first two or three are sufficient for separating the groups, which give a simple graphical representation, each sample having a value on each canonical variate these being plotted on co-ordinate axes. The group separations can be seen immediately and stray observations picked out. Not all of the thirteen measurements may be used in the analysis.

The (sample) Mahalanobis distance between two groups is the square root of

$$(\underset{\sim}{\bar{x}}_1 - \underset{\sim}{\bar{x}}_2)^T \underset{\sim}{S}^{-1} (\underset{\sim}{\bar{x}}_1 - \underset{\sim}{\bar{x}}_2)$$

where $\underset{\sim}{\bar{x}}_1$, and $\underset{\sim}{\bar{x}}_2$ are the (vector) sample means of the two groups and is a matrix of elements describing the variability of all the measurements (sample variance-covariance matrix). The SPSS 'MAHAL' option selects the amino acids one at a time, calculates the Mahalanobis distance between each pair of groups and finds the minimum value. This is then compared with the default value of 1.0 to enter without the new variable and if it has not increased, this new variable is discarded (Gnandesikan, 1977 and Kendall, Stuart and Ord, 1976).

(b) A Typical SPSS Control File for the U.K. Survey Honeys

Given below is an example of a typical Statistical Package for Social Sciences (SPSS) control file. This particular SPSS control file contained seventy four control statements or cards. Each control statement of card is divided into two distinct fields. A field being a part of a statement or a line which is composed of eighty characters or columns. The control field contains the command names starting from column one and ends at column fifteen of a line. The specification field contains the extra information needed for the command to operate the routine or functions defined by the control field. This specification field starts from column sixteen and ends at column eighty of a line.

The control statements of an SPSS control file are divided into three types. These are data definition, task definition and read data statements.

The data definition statements inform and describes the data to the SPSS. In this case, the procedure name denoted as Run Name, the list of variables, the file which contains the raw-input data, the structure of the data for each honey sample and the location of each variable on them, the number of honey samples, the labels used to define each variable, the numerical codes used for identifying each of the variables and the missing values for incomplete data.

The task definition statements activate, define and control the calculations to be performed on the data. In this case, the original amino acid concentration values that were utilized required to be coded. This was due to the fact that the amount of work space specified by these concentration values for each of the amino acids could not be handled by the SPSS. Hence, the concentration values were coded into four arbitary values 1, 2, 3 and 4. For example, the control statement 43 specifies to the SPSS that the concentration values between 50 and 99 nMoles per g of honey, inclusive, be allocated a value of 1. Similarly, for the other concentration range values. The samples containing the following pollens which were classed as predominant were selected into nine groups by the nine 'IF' control statements. These nine pollen groups were: *Brassica, Trifolium repens, Castanea, Calluna, Myosotis, Aesculus, Impatiens, Vicia* and *Clemantis*. These selected samples of honey were then allocated into their appropriate pollen groups labelled numerically from one to nine in the order given above. Canonical variates analysis was performed using the subprogram 'DISCRIMINANT' on the nine pollen groups using the arbitary codes for the amino acid concentration values. The 'MAHAL' method was used to maximise Mahalanobis distance between the pollen groups. The discrimination analysis and the Mahalanobis distance have been defined above, in part (a). A stepwise selection procedure was used and was defined as a default value, that is, twice the number of variables listed in the analysis. This stepwise selection procedure is where variables are either entered or removed from each separate step of the analysis. The number of discriminant functions derived were three and the minimum cumalative percentage was defined as 100%. A maximum value of 1.0 was defined for the significance levels of additional functions. The prior probability, that is, assigning the honey sample to that group for which it has the greatest probability of membership was defined as the value, equal for all the groups. The details of the processing options and optional statistics used are detailed extensively by Nie et al.,(1975).

The 'READ INPUT DATA' statement instructs the SPSS to begin reading the data from the raw-input data file in this case named as 'DAT1-UK1'.

The structure of this SPSS control file has been detailed below in the order required by the SPSS which was typed in at a visual display unit terminal.

```
 1) RUN NAME        A TEST OF DISCRIMINANT
 2) VARIABLE LIST   AA1 TO AA8
 3)                 AA9 TO AA13
 4)                 Q1 TO Q3, P1 TO P9, BFC
 5)                 A1 TO A17
 6) INPUT MEDIUM    [DAT1-UK1]
 7) INPUT FORMAT    FIXED(8F10.2/5F10.2/
 8)                 F3.0,2X,2(F2.0,3X),3(F3.0,2X,F5.1),2F5.1,F1.0,2X,F3.0/
 9)                 17F3.0)
10) NO OF CASES     192
11) VAR LABELS      AA1,LYS/AA2,ASP/AA3,THR/AA4,SER/AA5,GLU/
12)                 AA6,PRO/AA7,GLY/AA8,ALA/AA9,VAL/AA10,ILE/
13)                 AA11,LEU/AA12,TYR/AA13,PHE/
14)                 Q1,DATE OF REMOVAL OF HONEY FROM COMB/
15)                 Q2,HAS THE HONEY BEEN HEATED? YES OR NO/
16)                 Q3,HAS THIS COLONY BEEN FED SUGAR? YES OR NO/
17)                 P1,PREDOMINANT POLLEN>45%
18)                 P2,PERCENTAGE OF P1/
19)                 P3,SECONDARY POLLEN 16% - 45%/
```

```
20)                 P4,PERCENTAGE OF P3/
21)                 P5,SECONDARY POLLEN 16% - 45%/
22)                 P6,PERCENTAGE OF P5/
23)                 P7,PERCENTAGE OF UNIDENTIFIED POLLEN/
24)                 P8,PERCENTAGE OF SHRIVELLED POLLEN/
25)                 P9,POLLEN CONTENT/
26)                 BFC,ENGLISH HONEY OR FOREIGN AND COMMERCIAL HONEY/
27)                 A1,α-ABU/A2,γ-ABU/A3,ARG/A4,ASN/
28)                 A5,CYS/A6,GLUC/A7,GLN/A8,HIS/A9,MET/
29)                 A10,TRP/A11,HPRO/A12,HPIP/A13,MEHIS/A14,β-ALA/
30)                 A15,PIP/A16,p/A17,X/
31) VALUE LABEL     Q1 (101)JANUARY (102)FEBRUARY (103)MARCH (104)APRIL
32)                 (105)MAY (106)JUNE (107)JULY (108)AUGUST (109)SEPTEMBER
33)                 (110)OCTOBER (111)NOVEMBER (112)DECEMBER 1981
34)                 (201)JANUARY (202)FEBRUARY (203)MARCH (204)APRIL
35)                 (205)MAY (206)JUNE (207)JULY (208)AUGUST (209)SEPTEMBER
36)                 (210)OCTOBER (211)NOVEMBER (212)DECEMBER 1982
37)                 Q2 (88) YES (99) NO (0) UNKNOWN
38)                 Q3 (88) YES (99) NO (0) UNKNOWN
39)                 P1 (999) NOT PRESENT P3 (999) NOT PRESENT P5 (999) NOT
                    PRESENT
40)                 P2 (100) NOT PRESENT P4 (100) NOT PRESENT P6 (100) NOT
                    PRESENT
41)                 P9 (1) VERY LOW OR ZERO (2) LOW (3) MEDIUM (4) HIGH (5)
                    VERY HIGH (0) UNKNOWN
42)                 A1 TO A18 (66) PRESENT (77) NOT PRESENT (00) UNKNOWN
43) RECODE          AA1 TO AA5(50 THRU 99=1)
44)                           (99 THRU 249=2)
45)                           (249 THRU 499=3)
46)                           (499 THRU HIGHEST=4)
47) RECODE          AA7 TO AA3(50 THRU 99=1)
48)                           (99 THRU 249=2)
49)                           (249 THRU 499=3)
50)                           (499 THRU HIGHEST=4)
51) RECODE          AA6(1000 THRU 1999=1)
52)                    (1999 THRU 3999=2)
53)                    (3999 THRU 5499=3)
54)                    (5499 THRU HIGHEST=4)
55)  IF             (P1 EQ 301) POLLEN=1
56) IF              (P1 EQ 308) POLLEN=2
57) IF              (P1 EQ 318) POLLEN=3
58) IF              (P1 EQ 319) POLLEN=4
59) IF              (P1 EQ 328) POLLEN=5
60) IF              (P1 EQ 305) POLLEN=6
61) IF              (P1 EQ 304) POLLEN=7
62) IF              (P1 EQ 309) POLLEN=8
63) IF              (P1 EQ 326) POLLEN=9
64) MISSING VALUE   AA1 TO AA13 (0)/Q1 TO Q3(0)/P1 TO P9(0)/A1 TO A17(0)/
65) DISCRIMINANT    GROUPS=POLLEN(1.,9.)/VARIABLES=AA1 TO AA13/
66)                 ANALYSIS=AA1 TO AA13/
67)                 METHOD=MAHAL/
68)                 MAXSTEPS=26/
69)                 FUNCTIONS=3,100.,1./
70)                 PRIORS=EQUAL/
71) OPTIONS         5,6,7,8,9,10,11,12,15,18,19
72) STATISTICS      1,2,3,4,5,6,7,8,9
73) READ INPUT DATA
74) FINISH
```

In the above list of control statements, the 'VARIABLE LIST' statements 2 to 5, have been defined in the 'VAR LABELS' statements 11 to 30. The numerical codes used for identifying some of the variables have been defined in the 'VALUE LABEL' statements 31 to 42. Further, the numerical codes used for identifying the pollen grains have been given in Table 30. The structure of the data in the data file 'DAT1-UK1' have been defined in the 'INPUT FORMAT' statements 7 to 9, which can be read by the SPSS.

(c) Modification of the SPSS Control File Listed in Part (b)

Given below is the modified structure of the SPSS control file given above in part (b). This modified part of the SPSS control file contained modifications which improved the discrimination between the pollen groups. The SPSS control file listed in part (b) was modified from control statement 43 onwards as listed below. These modifications were to transform the original amino acid concentration values into their square roots and are specified by control statements 43 to 55.

To select honey samples which contained pollens whose percentage presence in each of the honey samples was greater than 70%, the 'IF' control statements 56 to 60 were used for the following pollens. These pollens were: the *Brassica, Trifolium repens, Castanea, Calluna,* and *Myosotis*. By using the control statement 61, 'SET = TRUNC(UNIFORM(2.0))', the uniform (2.0) generates uniformly distributed random numbers between 0 and 2.0. Of these numbers so generated, 50% can be expected to be between 0 and 1.0. The use of the trunc function will give 0's for these numbers and 1's for the others. Therefore, SET will be a random variable in which the expected probability of the value 0 is 0.5 or some number close to it. The SELECT = SET(0) specifies that only those honey samples for which a set have a value of 0 will be used to compute discriminant function coefficients, but all the samples will be classified. The other honey samples for which SET has a value of 1, these will be used for estimating the misclassification rate (Hadlaihull and Nie, 1981).

Some of the processing options specified in the SPSS control file listed in part (b) were omitted and others were included. This removed unnecessary analysis not required in this case and introduced other which were required. Details of these options are given by Nie et al., (1975).

The structure of the modified section of the SPSS control file given in part (b) above has been listed below:

```
43) COMPUTE          LYS=SQRT(AA1)
44) COMPUTE          ASP=SQRT(AA2)
45) COMPUTE          THR=SQRT(AA3)
46) COMPUTE          SER=SQRT(AA4)
47) COMPUTE          GLU=SQRT(AA5)
48) COMPUTE          PRO=SQRT(AA6)
49) COMPUTE          GLY=SQRT(AA7)
50) COMPUTE          ALA=SQRT(AA8)
51) COMPUTE          VAL=SQRT(AA9)
52) COMPUTE          ILE=SQRT(AA10)
53) COMPUTE          LEU=SQRT(AA11)
54) COMPUTE          TYR=SQRT(AA12)
55) COMPUTE          PHE=SQRT(AA13)
56) IF               (P1 EQ 301 AND P2 GT 70.0) POLLEN=1
57) IF               (P1 EQ 308 AND P2 GT 70.0) POLLEN=2
58) IF               (P1 EQ 318 AND P2 GT 70.0) POLLEN=3
59) IF               (P1 EQ 319 AND P2 GT 70.0) POLLEN=4
60) IF               (P1 EQ 328 AND P2 GT 70.0) POLLEN=5
61) COMPUTE          SET=TRUNC(UNIFORM(2.0))
62) DISCRIMINANT     GROUPS=POLLEN(1.,5.)/VARIABLES=LYS TO PHE/
63)                  SELECT=SET(0)/
64)                  ANALYSIS=LYS TO PHE/
65)                  METHOD=MAHAL/
```

```
66)                 MAXSTEPS=26/
67)                 FUNCTIONS=3,100.,1./
68)                 PRIORS=EQUAL/
69) OPTIONS         2,3,5,6,7,8,10,11,12
70) STATISTICS      1,2,3,4,5,6,7,8,9
71) READ INPUT DATA
72) FINISH
```

(d) Control Statements of Calendar Year 1981 and 1982, and the Five Main Pollens - *Brassica, Trifolium repens, Castanea, Calluna* and *Myosotis*

(i) Given below is the structure of control statements which were used for recoding the numerical codes that were used for distinguishing between the twelve calendar months. All the months of the calendar year 1981 and 1982 were recoded to represent each year. These control statements listed below replaced the control statements 56 to 60 of the SPSS control file given in part (c) above;

```
56) RECODE      Q1(101=112)      69) RECODE      Q1(202=212)
57) RECODE      Q1(102=112)      70) RECODE      Q1(203=212)
58) RECODE      Q1(103=112)      71) RECODE      Q1(204=212)
59) RECODE      Q1(104=112)      72) RECODE      Q1(205=212)
60) RECODE      Q1(105=112)      73) RECODE      Q1(206=212)
61) RECODE      Q1(106=112)      74) RECODE      Q1(207=212)
62) RECODE      Q1(107=112)      75) RECODE      Q1(208=212)
63) RECODE      Q1(108=112)      76) RECODE      Q1(209=212)
64) RECODE      Q1(109=112)      77) RECODE      Q1(210=212)
65) RECODE      Q1(110=112)      78) RECODE      Q1(211=212)
66) RECODE      Q1(111=112)      79) RECODE      Q1(212=212)
67) RECODE      Q1(112=112)      80) IF          (Q1 EQ 112) YEAR=1
68) RECODE      Q1(201=212)      81) IF          (Q1 EQ 212) YEAR=2
```

(ii) Given below are the modifications of the control statements 79 and 80 in the above list and were replaced by those given below to specify:

1. *Brassica* pollen

```
79) IF          (Q1 EQ 112 AND P1 EQ 301) YEAR=1
80) IF          (Q1 EQ 212 AND P1 EQ 301) YEAR=2
```

2. *Trifolium repens* pollen

```
79) IF          (Q1 EQ 112 AND P1 EQ 308) YEAR=1
80) IF          (Q1 EQ 212 AND P1 EQ 308) YEAR=2
```

3. *Castanea* pollen

```
79) IF          (Q1 EQ 112 AND P1 EQ 318) YEAR=1
80) IF          (Q1 EQ 212 AND P1 EQ 318) YEAR=2
```

4. *Calluna* pollen

```
79) IF          (Q1 EQ 112 AND P1 EQ 319) YEAR=1
80) IF          (Q1 EQ 212 AND P1 EQ 319) YEAR=2
```

5. *Myosotis* pollen

```
79) IF          (Q1 EQ 112 AND P1 EQ 328) YEAR=1
80) IF          (Q1 EQ 212 AND P1 EQ 328) YEAR=2
```

(e) Control Statements of Heating and Sugar Feeding

Given below are modifications of the control statements 79 to 80 from (d) to specify:

(i) Heating

```
79) IF          (Q2 EQ 88) HEAT=1
80) IF          (Q2 EQ 99) HEAT=2
```

(ii) Sugar feeding

```
79) IF          (Q3 EQ 88) SUGAR=1
80) IF          (Q3 EQ 99) SUGAR=2
```

(f) Control Statements of the Four Combination of Heated and Sugar-fed Groups

Given below is the list of control statements which specified the four various combinations of the two questionnaire questions number 5 and 6 into two groups, that is, 'HEATSF1 and 2'. These control statements were used to replace the 'IF' control statements 56 to 60 given in part (c) above.

(i) the 'heated and sugar-fed' group and the 'not heated and not sugar-fed' groups:

```
56) IF          (Q2 EQ 88 AND Q3 EQ 88) HEATSF=1
57) IF          (Q2 EQ 99 AND Q3 EQ 99) HEATSF=2
```

(ii) the 'heated and not sugar-fed' group and the 'not heated and sugar-fed' groups:

```
56) IF          (Q2 EQ 88 AND Q3 EQ 99) HEATSF=1
57) IF          (Q2 EQ 99 AND Q3 EQ 88) HEATSF=2
```

(iii) the 'heated and not sugar-fed' group and the 'not heated and not sugar-fed' groups:

```
56) IF          (Q2 EQ 88 AND Q3 EQ 99) HEATSF=1
57) IF          (Q2 EQ 99 AND Q3 EQ 99) HEATSF=2
```

(iv) the 'heated and sugar-fed' group and the 'not heated and sugar-fed' groups:

```
56) IF          (Q2 EQ 88 AND Q3 EQ 88) HEATSF=1
57) IF          (Q2 EQ 99 AND Q3 EQ 88) HEATSF=2
```

APPENDIX VII

DISCRIMINANT ANALYSIS OF THE FOREIGN AND COMMERCIAL HONEYS

(a) A Typical SPSS Control File for the Foreign and Commercial Honeys

Given below is an SPSS control file that was used to discriminante between country groups from the foreign and commercial honey samples. This SPSS control file is similar in structure to that listed in Appendix VI (b). The only differences are in the 'NUMBER OF CASES', 'VAR LABELS', 'VALUE LABELS' and 'IF' control statements. In this case the 'IF' control statements 54 to 58 specified the SPSS to select the following countries. These countries were: Australia, Canada, Mexico, New Zealand and England commercial.

The structure of the SPSS control file is listed below in the order required by the SPSS which was typed in at a visual display unit terminal.

```
 1) RUN NAME        A TEST OF DISCRIMINANT ON FOREIGN AND COMMERCIAL HONEYS
 2) VARIABLE LIST   AA1 TO AA8
 3)                 AA9 TO AA13
 4)                 Q1 TO Q3, P1 TO P9, BFC
 5)                 A1 TO A17
 6) INPUT MEDIUM    [FC1]
 7) INPUT FORMAT    FIXED(8F10.2/5F10.2/
 8)                 F3.0,2X,2(F2.0,3X),3(F3.0,2X,F5.1),2F5.1,F1.0,2X,F3,0/
 9)                 17F3.0)
10) NO OF CASES     64
11) VAR LABELS      AA1,LYS/AA2,ASP/AA3,THR/AA4,SER/AA5,GLU/
12)                 AA6,PRO/AA7,GLY/AA8,ALA/AA9,VAL/AA10,ILE/
13)                 AA11,LEU/AA12,TYR/AA13,PHE/
14)                 Q1,COUNTRY OF ORIGIN/
15)                 Q2,/
16)                 Q3,HAS THIS SAMPLE OF HONEY BEEN COMMERCIALLY PROCESSED?
                    YES OR NO/
17)                 P1,PREDOMINANT POLLEN>45%/
18)                 P2,PERCENTAGE OF P1/
19)                 P3,SECONDARY POLLEN 16% - 45%/
20)                 P4,PERCENTAGE OF P3/
21)                 P5,SECONDARY POLLEN 16% - 45%/
22)                 P6,PERCENTAGE OF P5/
23)                 P7,PERCENTAGE OF UNIDENTIFIED POLLEN/
24)                 P8,PERCENTAGE OF SHRIVELLED POLLEN/
25)                 P9,POLLEN CONTENT/
26)                 BFC,ENGLISH HONEY OR FOREIGN AND COMMERCIAL HONEY.
27)                 A1,α-ABU/A2,γ-ABU/A3,ARG/A4,ASN/
28)                 A5,CYS/A6,GLUC/A7,GLN/A8,HIS/A9,MET/
29)                 A10,TRP/A11,HPRO/A12,HPIP/A13,MEHIS/A15,β-ALA/
30)                 A15,PIP/A16,p/A17,X/
31) VALUE LABEL     Q1(001)AUSTRIA (002)AUSTRALIA (003)ARGENTINA (004)CANADA
32)                 (005)CAYMAN ISLAND (006)CHINA (007)CHILE (008)FRANCE
33)                 (009)GREECE (010)HUNGARY (011)INDIA (012)MALTA
34)                 (013)MEXICO (014)NEW ZEALAND (015)RUSSIA (016)SPAIN
35)                 (017)UNKNOWN COUNTRY ORIGIN (018)ENGLISH (019)POTATO
                    STARCH (020)GOLDEN SYRUP
36)                 Q3, (44) PROCESSED (55) NOT PROCESSED (00) UNKNOWN
37)                 P1 (999) NOT PRESENT P3 (999) NOT PRESENT P5 (999)
                    NOT PRESENT
38)                 P2 (100) NOT PRESENT P4 (100) NOT PRESENT P6 (100)
                    NOT PRESENT
```

```
39)                P9 (1) VERY LOW OR ZERO (2) LOW (3) MEDIUM (4) HIGH
                   (5) VERY HIGH (0) NOT POLLEN
40)                A1 TO A17 (66) PRESENT (77) NOT PRESENT (00) UNKNOWN
41) COMPUTE        LYS=SQRT(AA1)
42) COMPUTE        ASP=SQRT(AA2)
43) COMPUTE        THR=SQRT(AA3)
44) COMPUTE        SER=SQRT(AA4)
45) COMPUTE        GLU=SQRT(AA5)
46) COMPUTE        PRO=SQRT(AA6)
47) COMPUTE        GLY=SQRT(AA7)
48) COMPUTE        ALA=SQRT(AA8)
49) COMPUTE        VAL=SQRT(AA9)
50) COMPUTE        ILE=SQRT(AA10)
51) COMPUTE        LEU=SQRT(AA11)
52) COMPUTE        TYR=SQRT(AA12)
53) COMPUTE        PHE=SQRT(AA13)
54) IF             (Q1 EQ 002) COUNTRY=1
55) IF             (Q1 EQ 004) COUNTRY=2
56) IF             (Q1 EQ 013) COUNTRY=3
57) IF             (Q1 EQ 014) COUNTRY=4
58) IF             (Q1 EQ 018) COUNTRY=5
59) COMPUTE        SET=TRUNC(UNIFORM(2.0))
60) DISCRIMINANT   GROUPS=COUNTRY(1.,5.)/VARIABLES=LYS TO PHE/
61)                SELECT=SET(0)/
62)                ANALYSIS=LYS TO PHE/
63)                METHOD/MAHAL/
64)                MAXSTEPS=26/
65)                FUNCTIONS=3,100.,1./
66)                PRIORS=EQUAL/
67) OPTIONS        2,3,5,6,7,8,10,11,12
68) STATISTICS     1,2,3,4,5,6,7,8,9
69) READ INPUT DATA
70) FINISH
```

(b) Control Statements of the Country, Floral Source, Pollen Types and Commercial Processing

Listed below are the control statements which were used for replacing the control statements 54 to 58 of the SPSS control file given in Part (a) above.

(i)
```
54) IF    (Q1 EQ 002) COUNTRY=1
55) IF    (Q1 EQ 004) COUNTRY=2
56) IF    (Q1 EQ 013) COUNTRY=3
57) IF    (Q1 EQ 014) COUNTRY=4
```

(ii)
```
54) IF    (Q1 EQ 002) COUNTRY=1
55) IF    (Q1 EQ 004) COUNTRY=2
56) IF    (Q1 EQ 006) COUNTRY=3
57) IF    (Q1 EQ 013) COUNTRY=4
58) IF    (Q1 EQ 014) COUNTRY=5
```

(iii)
```
54) IF    (Q1 EQ 002) COUNTRY=1
55) IF    (Q1 EQ 004) COUNTRY=2
56) IF    (Q1 EQ 006) COUNTRY=3
57) IF    (Q1 EQ 008) COUNTRY=4
58) IF    (Q1 EQ 013) COUNTRY=5
59) IF    (Q1 EQ 014) COUNTRY=6
```

(iv)
```
54) IF    (Q1 EQ 002) COUNTRY=1
55) IF    (Q1 EQ 004) COUNTRY=2
56) IF    (Q1 EQ 006) COUNTRY=3
57) IF    (Q1 EQ 008) COUNTRY=4
58) IF    (Q1 EQ 011) COUNTRY=5
```

(v)
```
54) IF    (Q1 EQ 008) COUNTRY=1
55) IF    (Q1 EQ 016) COUNTRY=2
56) IF    (Q1 EQ 018) COUNTRY=3
```

(vi)
```
54) RECODE    Q1(009=001)
55) RECODE    Q1(016=008)
56) IF        (Q1 EQ 001) HONEY=1
57) IF        (Q1 EQ 008) HONEY=2
```

```
(vii) 54) IF        (P1 EQ 308) POLLEN=1
      55) IF        (P1 EQ 337) POLLEN=2

(viii) 54) IF       (P1 EQ 308) POLLEN=1
       55) IF       (P1 EQ 337) POLLEN=2
       56) IF       (P1 EQ 325) POLLEN=3

(ix) 54) IF         (Q3 EQ 44)  PROCESS=1
     55) IF         (Q3 EQ 55)  PROCESS=2
```

(c) Control Statements used for Distinguishing Between United Kingdom and Foreign Honeys

```
(i) 54) IF          (BFC EQ 000) COUNTRY=1
    55) IF          (BFC EQ 001) COUNTRY=2

(ii) 54) IF         (BFC EQ 000) COUNTRY=1
     55) IF         (Q1 EQ 002)  COUNTRY=2
     56) IF         (Q1 EQ 004)  COUNTRY=3
     57) IF         (Q1 EQ 006)  COUNTRY=4
     58) IF         (Q1 EQ 009)  COUNTRY=5
     59) IF         (Q1 EQ 011)  COUNTRY=6
     60) IF         (Q1 EQ 018)  COUNTRY=7
```

APPENDIX VIII

IDENTIFIED POLLEN GRAINS

Pollen grains that were identified, as described, in the two hundred and fifty six samples of honey have been listed below. The identity of each grain was determined at three levels of classification, namely plant family, genus and species, in that order. The species of pollen for each genus are given as those most likely to be present according to those quoted by Sawyer (1975 and 1981) and Crane, Walker and Day (1984). Common English names equivalent of the Latin botanical names have also been included for reference.

TABLE VIII

List of Pollen grains identified in the U.K. survey and the foreign and commercial honey samples, and compared with those reported by Sawyer (1975 and 1981) and Crane, Walker and Day (1984)

Plant Family[1]	Genus[2] (Botanical name)	Species[3a] (English name)	Comment
U.K. Survey honey samples			
Aceraceae	*Acer*	*pseudoplatanus* Sycamore	✓ MT
Aquifoliaceae	*Ilex*	*aquifolium* Holly	MT
Balsaminaceae	*Impatiens*	*glandulifera* Policeman's Helmet	MT
Betulaceae	*Alnus*	*glutinosa* Alder	
Boraginaceae	*Myosotis*	*Arvensis* Forget-me-not	MT
Caprifoliaceae	*Sambucus*	*nigra* Elder	MT
Compositae	*Carduus*	*acanthoides* Welted Thistle	
"	*Chrysanthemum*	- Ox-eye daisy	
"	*Taraxacum*	*officinale* Dandelion	✓
Cruciferae	*Brassica**	*napus* Rape	✓ MT
Ericaceae	*Calluna*	*vulgaris* Heather (ling)	✓ MT
Fagaceae	*Castanea*	*sativa* Sweet chestnut	✓ MT
"	*Quercus*	*robur* English oak	
Guttiferae	*Hypericum*	*caliciuum* Rose of Sharon	
Hippocastanaceae	*Aesculus*	*hippocastanum* Horse chestnut	✓
Labiate	*Teucrium*	*scorodonia* Wood sage	
Leguminosae	*Lotus*	*corniculatus* Birdsfoot Trefoil	✓

Cont'd.....

KEY

1, 2, 3 - The names below each title is according to Crane, Walker and Day, 1984 and Sawyer, 1981.

a - For reference to other possible plant species as honey sources of the same genus refer to Crane, Walker and Day, 1984.

* - For reference to other speices of *Brassica* refer to Table 7.0.

✓ - Data on chemical and physical composition of Unifloral honeys of this pollen source have been given by Crane, Walker and Day, 1984.

- Data on only the physical composition of Unifloral honeys of this pollen source have been given by Crane, Walker and Day, 1984.

MT - Main types of pollen found in honeys according to Sawyer 1975 and 1981.

R - Rare types of pollen found in honeys according to Sawyer 1975 and 1981.

HD - Honeydew honey source.

Table VIII cont'd.....

Plant Family[1]	Genus[2] (Botanical name)	Species[3a] (English name)	Comment
Leguminosae	*Melilotus*	*alba* White Melilot	✓
"	*Onobrychis*	*viciifolia* Sainfoin	✓ R
"	*Trifolium*	*incarnatum* Crimson Clover	✓
"	"	*pratense* Red Clover	✓ MT
"	"	*repens* White Clover	✓ MT
"	*Vicia*	*faba* Field Bean	+ MT
Liliaceae	*Allium*	- Lily	
"	*Endymion*	*non-scriptus* Bluebell	
"	*Lilium*	*regale* Royal Lily	
Malvaceae	*Malva*	*sylvestris* Mallow	
Oleaceae	*Ligustrum*	*vulgare* Privet	MT
Onagraceae	*Epilobium*	*augustifolium* Rosebay	✓
Pinaceae	*Pinus*	*sylvestris* Scots Pine	✓ HD
Polemoniaceae	*Phlox*	*drummondii* Phlox	
Polygonaceae	*Fagopyrum*	*esculentum* Buckwheat	✓ R
Ranunculaceae	*Clematis*	*vitalba* Traveller's Joy	
"	*Helleborus*	*niger* Christmas Rose	
Rosaceae	*Cotoneaster*	- Cotoneaster	
"	*Fragaria*	*x ananassa* Strawberry	
"	*Malus*	*domestica* Apple	+
"	*Prunus*	*domestica* Plum	MT
"	*Pyrus*	*communis* Pear	
"	*Rubus*	*fruticosus* Blackberry	+ MT
Salicaceae	*Salix*	*caprea* Sallow (Willow)	+
Scrophulariacea	*Linaria*	*vulgaris* Common Toadflax	
Tiliaceae	*Tilia*	*cordata* Lime	✓ MT
Umbelliferae	*Heracleum*	*sphondylium* Hogweed	
Urticaceae	*Urtica*	*dioica* Nettle	
Violiaceae	*Viola*	*tricolor* Pansy	
English Commercial Samples			
Boraginaceae	*Echium*	*vulgare* Viper's Bugloss	✓ R
Compositae	*Helianthus*	*annuus* Sunflower	✓ R
Cruciferae	*Brassica*	*vapus* Rape	✓ MT
Ericaceae	*Calluna*	*vulgaris* Heather (ling)	✓ MT
Fagaceae	*Castanea*	*sativa* Sweet Chestnut	✓ MT
Labialae	*Rosmarinus*	*officinalis* Rosemary	✓
Leguminosae	*Lotus*	*corniculatus* Birdsfoot Trefoil	✓
"	*Robinia*	*pseudoacacia* False Acacia	✓
"	*Trifolium*	*pratanse* Red Clover	✓ MT
"	"	*repens* White Clover	✓ MT
"	*Vicia*	*faba* Field Bean	✓ MT
Myrtaceae	*Eucalyptus*	- Eucalyptus	✓ R
Tiliaceae	*Tilia*	*cordata* Lime	✓ MT

Cont'd.....

Table VIII cont'd.....

Plant Family[1]	Genus[2] (Botanical name)	Species[3a] (English name)		Comment
Foreign Samples				
		America's		
Argentina				
Compositae	*Cirsium*	-	Thistle	
Cruciferae	*Brassica*	*napus*	Rape	✓
Myrtaceae	*Eucalyptus*	-	Eucalyptus	✓ MT
"	-	-	-	MT
Canada				
Boraginaceae	*Echium*	*vulgare*	Viper's Bugloss	✓
Compositae	*Cirsium*	-	Thistle	MT
Cruciferae	*Brassica*	*napus*	Rape	✓ MT
Leguminosae	*Acacia*	-	Acacia	✓
"	*Melilotus*	-	Melilot	✓
"	*Trifolium*	*pratanse*	Red Clover	✓ MT
"	-	*repens*	White Clover	✓ MT
Myrtaceae	*Eucalyptus*	-	Eucalyptus	✓
"	-	-	-	
Cayman Island				
Bruseraceae	-	-	-	MT
Ericaceae	-	-	-	
Tiliaceae	*Tilia*	-	Lime	✓
Chile				
Boraginaceae	*Echium*	*plantagineum*	Purple Bugloss	✓
Bruseraceae	-	-	-	
Compositae	*Taraxacum*	-	Dandelion	✓
Labiatae	-	-	-	
Myrtaceae	-	-	-	
Mexico				
Bruseraceae	-	-	-	MT
Compositae	*Viguiera*	*helianthoides*	"Tah" Railway Daisy	+ MT
Leguminosae	*Acacia*	-	Wattle	✓ MT
"	*Robinia*	*pseudoacacia*	False Acacia	✓
Myrtaceae	*Eucalyptus*	-	Eucalyptus	✓
Polygonaceae	*Fagopyrum*	*esculentum*	Buckwheat	✓
Proteaceae	*Banksia*	*serrata*	Red Honeysuckle	✓
Tiliaceae	*Tilia*	-	Lime	✓

Cont'd.....

Table VIII cont'd.....

Plant Family[1]	Genus[2] (Botanical name)	Species[3a] (English name)		Comment
Asia				
China				
Compositae	*Cirsium*	-	Thistle	
"	*Taraxacum*	-	Dandelion	✓
Cruciferae	*Brassica*	*napus*	Rape	✓
Leguminosae	*Acacia*	-	Wattle	✓
"	*Lotus*	-	Birdsfoot Trefoil	✓
"	*Robinia*	*pseudoacacia*	False Acacia	✓
"	*Trifolium*	*pratanse*	Red Clover	✓
"	"	*repens*	White Clover	✓
"	*Vicia*	*faba*	Field Bean	+
Myrtaceae	*Eucalyptus*	-	Eucalyptus	✓
Polygonaceae	*Fagopyrum*	*esculentum*	Buckwheat	✓
Rutaceae	*Citrus*	-	Citrus	✓
Tiliaceae	*Tilia*	-	Lime	✓
Urticaceae	*Urtica*	-	Nettle	
Australia				
Australia				
Boraginaceae	*Echium*	*plantagenium*	Purple Bugloss	✓ MT
Cruciferae	*Brassica*	*napus*	Rape	✓ MT
Leguminosae	*Acacia*	-	Wattle	✓ MT
Myrtaceae	*Eucalyptus*	-	Eucalyptus	✓ MT
"	-	-	-	MT
Protenceae	*Banksia*	*serrata*	Red Honeysuckle	✓ MT
New Zealand				
Compositae	*Cirsium*	-	Thistle	MT
Cruciferae	*Brassica*	*napus*	Rape	✓ MT
Leguminosae	*Trifolium*	*repens*	White Clover	✓ MT
Myrtaceae	*Eucalyptus*	-	Eucalyptus	✓ MT
Russia				
Boraginaceae	*Echium*	*plantagenium*	Purple Bugloss	✓
Compositae	*Cirsium*	-	Thistle	
"	*Viguiera*	*helianthoides*	"Tah" Railway Daisy	+
Cruciferae	*Brassica*	*napus*	Rape	✓
Myrtaceae	*Eucalyptus*	-	Eucalyptus	✓

Cont'd.....

Table VIII cont'd.....

Plant Family[1]	Genus[2] (Botanical name)	Species[3a] (English name)		Comment
		Europe		
Austria				
Compositae	*Cirsium*	-	Thistle	
Ericaceae	*Erica*	-	Heather/Heath	✓
Fagaceae	*Castanea*	-	Chestnut	✓
France				
Compositae	*Helianthus*	*annuus*	Sunflower	✓
Cruciferae	*Brassica*	*napus*	Rape	✓
Fagaceae	*Castanea*	-	Sweet Chestnut	✓
Hippocastanaceae	*Aesculus*	-	Horse Chestnut	✓
Leguminosae	*Acacia*	-	Wattle	✓
"	*Trifolium*	*repens*	White Clover	✓
Rosaceae	*Prunus*	-	Fruit Blossom	
"	*Pyrus*	-		
Greece				
Compositae	*Centaurea*	*cyanus*	Cornflower	✓
Ericaceae	*Erica*	-	Heather/Heath	✓
Leguminosae	*Lotus*	*corniculatus*	Birdsfood Trefoil	✓
"	*Onobrychis*	-	Sainfoin	✓
Hungary				
Compositae	*Helianthus*	*annuus*	Sunflower	✓
"	*Taraxacum*	*officinale*	Dandelion	✓
Cruciferae	*Brassica*	*napus*	Rape	✓
Leguminosae	*Trifolium*	*repens*	White Clover	✓
"	*Robinia*	*pseudoacacia*	False Acacia	✓
Malta				
Labiatae	*Rosmarinus*	-	Rosemary	
Leguminosae	*Lotus*	*corniculatus*	Birdsfoot Trefoil	✓
"	*Melilotus*	-	Mililot	✓
Spain				
Compositae	*Cirsium*	-	Thistle	MT
"	*Helianthus*	*annuus*	Sunflower	✓
"	*Viguiera*	*helianthoides*	"Tah" Railway Daisy	+
Cruciferae	*Brassica*	*napus*	Rape	✓ MT
Leguminosae	*Robinia*	*pseudoacacia*	False acacia	✓
"	*Trifolium*	*pratense*	Red Clover	✓
"	"	*repens*	White Clover	✓ MT
Rutaceae	*Citrus*	-	Citrus	✓ MT
Salicaceae	*Salix*	*caprea*	Willow	

APPENDIX IX

LIST OF PUBLICATIONS

1. Croft, L.R., Mistry, R.P. and Washington, R.J. (1986) - 'Analysis of the Proteins in Honey by Gel Electrophoresis' (presented as poster session) at the 'Fifth Meeting of the International Electrophoretic Society' in London.

2. Mistry, R.P., Washington, R.J. and Croft, L.R. (1986) 'Correlation of Amino Acids with Pollen Analysis' (lecture) at the '1st Bee Research Workers Colloquia' in London.

3. Washington, R.J., Croft, L.R. and Mistry, R.P. (1986) 'Analytic Studies on Honey' International Bee Research Association, Cardiff.

POSSIBLE CONTRIBUTION TO BEE WORLD

At the first Bee Research Colloquium held at London on 19th February 1986, papers were read by R. J. Washington, R. P. Mistry and S. Slight on behalf of work carried out by the Department of Biological Sciences, University of Salford.

Since 1975, some 27 graduate students have been involved, under our supervision, with the analysis of hive products. The major emphasis has been on the amino acids of pollen and of honey (Mr. J. M. Carter), the relationship of amino acid patterns to floral source (Miss M. A. Siddiqui) and, most recently, quantitation of free amino acid levels with determined floral sources and computer predictions (Mr. R. P. Mistry). He also carried out a honey sample survey of UK honeys for the BBKA. This technique was shown to be feasible even for pollen-free samples.

Progress was also made on the characterisation of the protein responsible for thixotrophy in heather honey.

As well as an interest in the detection of adulteration and misrepresentation of honey, there is work progressing on the toxicity of HMF (Mr. S. Lee), a field which has been neglected. He showed that, in vitro, the upper limit of 5 mg HMF per ml of fluid prevented all protein synthesis.

The mechanism of action of bee venom, principally melittin, on lens protein synthesis is being investigated by Mr. S. Slight. He showed, in vitro, that levels of 10 ug per ml of bee venom injected, arrested protein synthesis in the eye lens and caused irreversible changes to lens structure, resulting in opacity.

R. J. Washington

L. R. Croft

Washington, R. J., Croft, L. R. and Mistry, R. P. (1986) Analysis of honey. Paper presented, London, 19th February

Croft, L. R., Washington, R. J. and Mistry, R. P. (1986) Final report: Analysis of honey. The British Beekeepers' Association Year Book, June, p20

Croft, L. R., Mistry, R. P. and Washington, R. J. (1986) Analysis of the proteins of honey by gel electrophoresis. 5th Int. Conf. on Electrophoresis, London, September (to be pub: Springer-Verlag)

I confirm the above is a true record of Mr. R. P. Mistry publications

R.J. Washington

20/7/87

ANALYSIS OF THE PROTEINS IN HONEY BY GEL ELECTROPHORESIS

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1 INTRODUCTION

The adulteration and misrepresentation of honey are major threats to the viability of the apicultural industry (1). At this present time honey may be subject to adulteration with high fructose syrups. Although these may be detected in honey by stable isotope analysis, the instrumentation necessary is extremely costly and not readily available to many public analysts (2). In addition the technique is not able to detect the presence of high fructose syrups prepared from C_3 plants (3).

On the other hand, misrepresentation of honey may be readily detected by melissopalynology (4). But this is not so if the honey, during processing, has been highly sieved so that most of the pollen has been removed.

Honey is known to contain approximately 0.5% protein. These include specific enzymes derived directly from the bee, hence reduced levels of these are a good indication that adulteration has occurred. Other proteins are also present. These, in the main, are derived from the pollen, or plant nectar, and so serve as the basis for the determination of the floral, and so, geographical origin of the honey.

Our procedure (5) involves analysis by PAGE-electrophoresis of protein concentrates derived from honey and is a simple and effective means for detecting adulteration and misrepresentation.

2 METHODS

The proteins present in a sample of honey are concentrated by ultrafiltration (Amicon Standard cell fitted with a UM10 membrane). This process, which is carried out at $4^{o}C$, is continued, after repeated dilution of the sample, until the filtrate is negative for sugar. The resulting, mainly protein concentrate is freeze-dried.

Polyacrylamide gel electrophoresis of the samples is conducted in the presence of sodium dodecyl sulphate (6). Gels are stained with Commassie Brilliant Blue and after destaining are scanned on an ISCO model 659 scanner.

3 RESULTS

Because of the low levels of proteins in honey it was found to be necessary to submit the samples to a preliminary concentration using ultrafiltration. Good separation of the proteins in the concentrates was achieved by electrophoresis in the presence of SDS, on 10% polyacrylamide gels.

In this way it was found that as many as eleven protein bands could be resolved in the honey samples. The presence or absence of any of these bands was used to characterize any particular honey sample with regard to its likely geographical origin.

The technique is also able to detect adulteration and in fact is so sensitive as to detect honeys that had been obtained from bees fed on pollen substitutes.

4 ACKNOWLEDGEMENT

We would like to thank the British Beekeepers' Association for financial support.

5 REFERENCES

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(5) R.P. Mistry, Ph.D. Thesis, University of Salford, 1986.

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